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Supplement:

Proteases and Antiproteases in the Lung

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Proteases and Antiproteases in the Lung

Prologue

This supplement to the AMERICAN REVIEW OF RESPIRATORY DISEASE consists of ideas and concepts that were selected for presentation at the Symposium on Proteases and Antiproteases in the Lung, held on May 10, 1981 at the American Lung Association American Thoracic Society Annual Meeting. The publication date was delayed for one year to permit all observations that had not been published to be accepted by refereed journals. Therefore all concepts presented in the symposium have survived critical review. In addition, the manuscripts include published or in-press observations that bring the respective areas up to date as of May 1982.

Selection of areas covered in the symposium was based on two factors. The first section of manuscripts was selected because it represents important breakthroughs in our understanding of protease-inhibitor interactions in the lungs or has filled an important void in understanding such interactions. This void included a virtual ignorance about accessibility of neutrophil enzymes to normal or inflamed lungs. The participants were selected in 1979–1980. If I had to reselect areas of great importance to protease-inhibitor balance in the lungs today, I would include the areas of macrophage elastase, neutrophil elastase in association with macrophages, and other areas that now are becoming clarified.

The second part of the symposium was designed to describe the program under way at the Lung Division of the National Heart, Lung and Blood Institute and in the academic community to use the basic information on the protease-inhibitor balance in the lung to bring about eventual clinical trials of new agents that may prevent the progression of emphysema. This subject was alluded to in an editorial published previously in this journal (1). In this supplement the reader can evaluate the progress that has been made and that must be made before therapeutic trials will become feasible.

Although I anticipate that my clinical colleagues will be interested in reading much of the exciting biology and the clinical trial plans, I can also assume they will find some of the chemistry beyond their areas of interest and knowledge. I can equally assume the reverse to be true for my chemically-oriented colleagues. However, because of the broad readership of the REVIEW, I have encouraged each participant to write "state of the science" papers in their respective areas and to include, insofar as possible, sufficient background information to permit the broader audience to get a feel for progress in their areas. In particular, I expect that the articles of Dr. Powers and Dr. Glaser, which are highly chemical in nature, will have critical importance to the pharmaceutical and blood fractionating industry as well as to the usual audience of this journal. If, taken as a whole, this symposium triggers some young mind to propose an important new experiment, or if it brings the clinical, research, and industrial communities from their Tower of Babel into further useful dialogue about these problems, it will be well worth the time and effort of the contributors.

A final comment will facilitate the reader's appreciation of the manuscripts. Investigators disagree on the best name for the protein of central interest in the symposium. It is called alpha-1-antitrypsin, alpha-1-proteinase inhibitor, alpha-1-protease inhibitor, and several other permutations of these names. In this regard, the manuscripts in the symposium accurately reflect the confusion in the literature, and the editors made no attempt to reconcile the warring factions.

ALLEN B. COHEN, *Editor*
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1. Cohen AB. Opportunities for the development of specific therapeutic agents to treat emphysema. Am Rev Respir Dis 1979; 120:723–7.

I. Neutrophils, Their Elastases and Inhibitors in the Lungs

Introduction

Understanding the functions of the lung, in terms of its tissue structural composition and the enzymatic constituents of its cells, is a subject of the last decade. A symposium with these titles could not have been conceived as little as six years ago. This is particularly true of the papers in this symposium focusing on clinical disease that may result from the enzymatic actions of neutrophils, macrophages, and platelets. We are just beginning to explore how cellular constituents of these cells injure capillary endothelium, alveolar epithelium, and basement membrane. As is evidenced by these papers and the general theme of this symposium, the critical questions being explored concern molecular interactions between proteases, and particularly elastases, and the structural components of the alveolar capillary membrane.

Although much has been learned of the cellular and tissue interactions that induce tissue injury, the onset of disease concerns the action of other factors, such as inhibitors and the tissue milieu which influence the response and modulate the injury. This regulation of tissue response may also distinguish acute injury from chronic states of imbalance which result in more profound anatomical changes in lung structure. These are areas of study which pose the prospect of not only clearer understanding of lung tissue injury in many different disease states, but also of devising therapies which prevent or control pathogenesis despite differences in the initiating agents.

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Neutrophils in Normal Lungs[1-2]

ALLEN B. COHEN and MICHAEL ROSSI

SUMMARY This report reviews the role of the lungs in the normal metabolism of neutrophils and the putative role of the neutrophil in lung metabolism. There are many apparent connections between the peripheral supply of neutrophils and the lungs. Neutrophils develop from primitive precursor cells in the bone marrow. Human alveolar macrophages secrete a factor that stimulates these precursors to proliferate. The neutrophils leave the marrow at carefully controlled rates to keep the concentration of peripheral neutrophils constant. Once the cells enter the circulation, they are rapidly distributed between the marginated and circulating pools. Together these pools comprise the total blood neutrophil pool. A large part of the marginated pool occurs in the lungs. Some agents that cause widespread sequestration of neutrophils in the lung capillaries may cause endothelial cell damage; however, normally the neutrophils leave the circulation relatively rapidly, with half lives from 3.5 to 7 h in different species. Inflammatory processes increase flux rates at every step to allow the accumulation of neutrophils at sites of inflammation.

Neutrophils enter the lungs at low rates in normal animals and humans. When neutrophils are stimulated to enter the lungs by repetitive lung washes, they enter the alveoli first by entering the interstitium of the lungs beneath Type II alveolar cells. They then appear to migrate through the alveolar basement membrane and exit between Type I and Type II cells, lifting fragments of the Type I cell with them into the alveoli. The neutrophil pool in normal rabbit air spaces is 6.1 × 10^6/g of lung tissue, and 0.38–0.41 of the pool turns over per hour.

Neutrophil migration into alveoli can be controlled by 2 chemotactic factors secreted by human alveolar macrophages. One appears to be a low molecular weight lipid-like substance and the other a peptide. In addition, the macrophages secrete an enzyme releasing factor that may be the same as the peptide chemotactic factor. This factor can release up to 12.5% of the neutrophil elastase from neutrophils that adhere to surfaces such as millipore membranes. The factor also stimulates the release of myeloperoxidase, another azurophilic granule constituent of neutrophils. The myeloperoxidase is capable of inactivating alpha-1-antitrypsin in the presence of hydrogen peroxide and halogen ions. The net interaction of these and other forces in the alveolus is unclear. As long as the alpha-1-antitrypsin is intact, the lung architecture is not destroyed with resultant emphysema. However, if the balance is tipped by smoking or by the genetic deficiency of alpha-1-antitrypsin, there appears to be sufficient neutrophil traffic in the otherwise normal lung to account for the lung destruction seen in these situations.

AM REV RESPIR DIS 1963; 127:S3–S9

Introduction

Investigators have implicated neutrophil proteases in a wide variety of human lung diseases. In particular, they have suggested that neutrophil proteases may destroy the lungs in individuals who lack the major serum proteinase inhibitor, alpha-1-antitrypsin (also called alpha-1-proteinase inhibitor), but who are otherwise normal. Therefore this report will address the question: Do neutrophils and their potentially toxic byproducts enter the normal lungs, and if so, how and why?

Evidence for the Role of Neutrophil Elastase in the Genesis of some Forms of Pulmonary Emphysema

It has been well documented that individuals who have very low blood concentrations of the serum proteinase inhibitor, alpha-1-antitrypsin, develop emphysema at an early age (1). Alpha-1-antitrypsin inhibits several human enzymes that are called elastases because of their ability to cleave native elastin. It has been established that elastases and neutrophil extracts from a number of species can produce emphysema-like pathologic (2–4) and physiologic (5–7) abnormalities in animal lungs. Janoff *et al* (8) showed that isolated human neutrophil elastase can produce emphysema-like lesions in dogs. Furthermore, alpha-1-antitrypsin has been demonstrated in alveolar macrophages and lung alveoli of humans (9–11), and neutrophils have been found in lung washes from normal humans, dogs, cows, and monkeys (12) (Cohen *et al*, 1979, unpublished observations) and in alveoli of fixed rabbit lungs (13). Hunninghake *et al* (14), on the other hand, found few neutrophils in normal human lung washes but many in washes from smokers. These data led investigators to formulate the hypothesis that emphysema in patients with a deficiency of alpha-1-antitrypsin may be caused by the unrestrained action of neutrophil elastase in the lungs. In addition, since the deficiency in alpha-1-antitrypsin is the primary defect in these patients, it seems likely that the inhibitor protects the lungs against damage from enzymes that occur in lungs of normal persons.

An association between alpha-1-antitrypsin deficiency and smoking recently was provided by studies showing that smoking reduced the functional elastase inhibitory capacity of humans (15, 16). In addition, Rodriguez *et al* (17) and Hinman *et al* (12) showed that alveolar macrophages from smokers secrete an elastase that may be neutrophil elastase which has been ingested by the macrophage. Taken together, these observations make a strong case for the role of neutrophils in the production of emphysema in smokers and alpha-1-antitrypsin deficient persons. It therefore becomes important to understand why neutrophils occur in the normal lung, what controls their influx, how the influx can be modulated, and what happens to the constituents of neutrophils that gain access to the lungs. These problems are the subject of this discussion.

Neutrophil Origin, Distribution and Fate in Normal Animals

Whereas many investigators have speculated about the ultimate fate of the neutrophil after it leaves the circulation, few studies of this phenomenon have been carried out, and to our knowledge no studies of the ultimate fate of lysosomal enzymes or other cellular constituents, i.e., membranes, have been carried out *in vivo* in the normal lung.

The discussion of neutrophils in normal lungs will be prefaced by a short discussion of neutrophils in the whole organism (figure 1). Neutrophil life cycles have been extensively studied in the last 2 decades. The kinetics of granulocyte production and release by the bone marrow have been extensively reviewed (18–20).

Neutrophils are derived from primitive precursors in the bone marrow. They enter a mitotic pool that is a conceptual pool in the bone marrow rather than a physical space. As they mature they migrate into a reserve pool (marrow neutrophil reserve pool) of relatively mature neutrophils that are readily accessible to the circulation. Neutrophils migrate into circulation on a regular basis to replace those leaving the circulation or to respond to an infection. These various functions of the bone marrow may be under humoral control under

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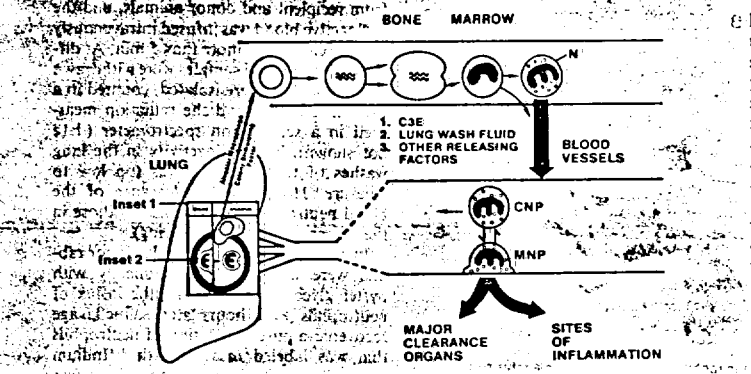

Fig. 1. Diagram of the life cycle of the neutrophil as it relates to the lungs. See text for details.

certain circumstances. Alveolar macrophages produce a "colony stimulating factor" that stimulates bone marrow cells *in vitro* to proliferate (21). Egress of neutrophils from the bone marrow can be stimulated by the C3e component of complement (22), or by reintroducing concentrated lung lavage back into the airways (23). In addition, the leukocytosis that occurs during recovery from vinblastine-induced leukopenia can be transferred by the plasma of the recovering animals (24).

The neutrophils enter the bloodstream and are immediately distributed between a marginated and a circulating pool. Together these pools comprise the total blood neutrophil pool. The marginated pool is demonstrated in the following fashion. Radioactively-labeled neutrophils are introduced into the blood from a donor. Within 5 minutes or less the labeled neutrophils are found to be diluted into a pool larger than the circulating neutrophil pool. If an agent that demarginates neutrophils, such as epinephrine, is then introduced into the circulation, these neutrophils rapidly re-enter the circulating pool of neutrophils. It has been demonstrated that one of the largest pools of marginated neutrophils is in the lungs.

After entry into the circulation, the neutrophils rapidly leave the circulation in a random fashion with a half-life of 3 to 7 hours depending on the species studied (25, 26). Once the neutrophils leave the circulation by attrition or by collecting at a site of infection, they do not return.

Role of the Lung in Neutrophil Clearance from Blood of Normal Animals

Although little was known until recently about the role of the lungs in the clearing of neutrophils in normal animals, it had been suggested that the lungs might be a major site of clearance of neutrophils from the circulation. The role of the lungs in clearance of neutrophils will be described in the following discussion.

Early studies of neutrophil flux in the lung were performed by Bierman and colleagues (27, 28). These investigators measured differences in neutrophil concentrations in pulmonary artery and peripheral arterial blood and found that the pulmonary circulation was capable of rapidly removing and delivering vast quantities of leukocytes from the marginated into the circulating neutrophil pool, as well as storing and destroying leukocytes (29). Respirations can rapidly change the number of neutrophils trapped in the lung (27), and, during cross-transfusion, the lung was capable of removing all of the infused leukocytes (27). However, the prior administration of heparin in doses up to 6 mg/kg made it possible for approximately 10 to 30% of the anticipated number of leukocytes to enter the arterial circulation following the transfusion of large numbers of leukocytes. With the return of the clotting toward normal, there was a prompt withdrawal of these leukocytes from the peripheral circulation. Withdrawal of leukocytes from the peripheral circulation was also initiated by the administration of histamine (29). Similar findings occurred following the administration of nicotinic acid, colloidal substances such as saccharated iron-oxide, plasmoid, and gelatin or heterospecific blood. In each instance, while the return to normal level usually occurred within a predictable time, there was no evidence of overcompensation with a redelivery of such withdrawn leukocytes back into the peripheral circulation. The control leukocyte level was reattained within 8 to 10 min after histamine administration in the normal animals, but about 60 min following nicotinic acid.

Recent Studies of Neutrophil Traffic in the Normal Lungs

Based on the studies reviewed above and others, investigators suggested that the lungs were a major clearance organ of neutrophils. However, lung morphologists assert that it is very difficult to find neutrophils in normal lungs. In our first series of studies, we examined the statement of Bierman and colleagues (27) that there was an arteriovenous difference of neutrophils across the lungs and then we measured the alveolar neutrophil pool size (13).

Animal preparation. Rabbits were anesthetized and a tracheostomy was performed (figure 2A). The rabbits were artificially ventilated for the short time (about 10 min) necessary to open the chest and draw right- and left-sided heart blood by direct cardiac puncture. They were then killed by administration of pentobarbital sodium. The heart and lungs were removed. The lungs, cannulated with two preweighed catheters, were divided and weighed. One lung was lavaged with six 25-ml aliquots of saline, 0.9%. Then both lungs were fixed in solution under a constant pressure of 25 cm formaldehyde.

Quantification of lung neutrophil pool size. The numerical density of particles or structures, in this case neutrophils (N_v, as neutrophils per unit lung volume), is related to the numerical density in a unit area (N_a, as neutrophils per unit area) of lung section by the following equation

$$N_v = N_a/D \qquad (1)$$

where D is the mean neutrophil diameter (17). If the subscripts w or u refer to data from the washed or unwashed lungs, respectively, then the following expression:

$$N_{v,w}/N_{v,u} = (N_{a,w}/D)/(N_{a,u}/D) = N_{a,w}/N_{a,u} \qquad (2)$$

equals the fraction of the initial number of neutrophils remaining in the washed lung. Therefore, the neutrophils in the lungs can be quantified by the equation

$$CVP/(1 - (N_w/a_w)/(N_u/a_u))1 + W_r/W_w1\ CVP/(1 - (N_w/a_w)/(N_u/a_u))1 \qquad (3)$$

where "C" is the concentration of cells in the lung wash, "V" is the volume of the lung wash, "P" is the percentage of cells in the lung wash that are neutrophils, "N" is the number of neutrophils counted in the alveoli of a lung section, "a" is the area in which the neutrophils were counted, and "W" is lung weight before lavage. To compute the number of neutrophils per rabbit, it was assumed that the number of air space neutrophils is proportional to the prewash weight of the lungs. The number of neutrophils per gram of lung was computed by dividing the number of lung neutrophils by the lung weight.

Quantification of air space neutrophils in the lungs. The results of these studies showed there were no significant differences in total leukocyte count or neutrophil concentration in right ventricular or left ventricular

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Fig. 2. Measurement of air space neutrophil pool size in rabbits. A. Morphometric analysis of alveolar neutrophil pool size. Because area densities are proportional to volume densities, we determined the ratios of the area densities of neutrophils in air spaces of washes and unwashed lungs and the number of neutrophils washed out. From this data we calculated the alveolar neutrophil pool size. B. Neutrophils in a normal alveolus of a rabbit (Original magnification: ×400).

blood drawn over 5 min. Samples from arterial and venous blood taken before anesthesia were similar to samples taken from the heart.

Although there was no discernible neutrophil removal by the lungs by examining arteriovenous differences across the lungs, there were clearly neutrophils on the air side of normal lungs (figure 2B).

Cell counts in lung washes of 8 animals are shown in table 1. Additional data were collected (n = 14) where only the percentage neutrophils in the lung wash and yield of neutrophils from the lung wash was obtained in animals killed without prior anesthesia or artificial ventilation. The mean percentage neutrophils was 2.13 ± 2.54 (SD)% and the mean recovery of neutrophils from the lavage fluid was $4.0 \times 10^8 \pm 4.44 \times 10^8$ (SD)/rabbit lung.

The fixed lungs were examined morphometrically as described above. The results are shown in table 2. These morphometric methods and the unique morphologic characteristics of neutrophils enabled us to measure the size of the pool of neutrophils in alveoli of rabbit lungs. Rabbit lungs contained an estimated 6.11×10^4 alveolar neutrophils/g wet wt of lungs, and 9.57×10^8 alveolar neutrophils/rabbit. Lung lavage was successful in removing an average of 42.2% of the neutrophils in the air-containing spaces of the lungs.

Turnover Time of Alveolar Neutrophils

The next series of experiments was designed to determine the turnover time of the alveolar neutrophil pool (30). In these experiments, radioactive neutrophils were injected into the blood of recipient rabbits. At intervals, the blood was drawn and the specific activity of the blood neutrophil was determined (figure 3).

One group of animals had their neutrophils labeled with ^{3}H-thymidine. Seventy-two hours later 30 ml of blood was drawn from recipient and donor animals, and the radioactive blood was infused intravenously in the recipient for more than 5 min. At different times, blood samples were withdrawn and neutrophils were isolated, counted in a Coulter Counter, and the radiation measured in a scintillation spectrometer (data not shown). The radioactivity in the lung washes of these animals was too low to measure. However, the clearance of the blood neutrophils was identical to those in the second group (see following).

In a second group of animals, donor rabbits were injected intraperitoneally with oyster glycogen to stimulate the influx of neutrophils. Four hours later, saline lavage recovered a pure population of neutrophils that was labeled *in vitro* with ^{111}Indium oxime (^{111}In). At intervals, the blood was sampled and the specific activity of the neutrophils was calculated from white blood cell concentration, differential counts, and radioactivity (figure 3).

At the end of each experiment, the indium-labeled animals were killed and the lungs washed with 0.9% sodium chloride. Total cell counts were measured and the percent of neutrophils determined. The curve of lung neutrophil specific activity is shown (figure 3). All radioactivity was in the cell pellet after centrifugation. The turnover time was measured by the relationship described by Zilversmit (31). The determination of the fractional turnover rate (k) (fraction of a pool turning over per unit time) was determined from 5 to 9 h (after steady state was reestablished) by the following relationship:

$$k = \frac{S_{A_{t_2}} - S_{A_{t_1}}}{\int_{t_1}^{t_2} (S_B - S_A)\, dt}$$

$$\frac{1}{k} = t_t$$

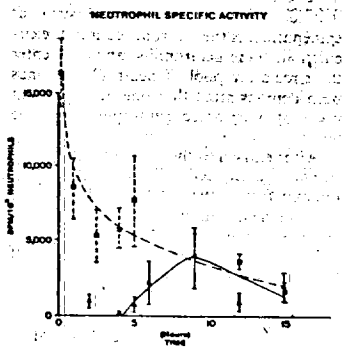

Fig. 3. Neutrophil turnover in blood and lungs. Blood neutrophil turnover was calculated from the curve of the change in specific activity of indium-labeled neutrophils in rabbit blood and lung washes. Blood neutrophil specific activity □ - - - - - □. Alveolar neutrophil specific activity Δ——Δ.

TABLE 1

LUNG WASH CELLS

	Total Cells Wash, × 10⁷	Neutrophils %	Lymphocytes %	Mononuclear Cells %
Mean	1.2	2.49	5.04	92.43
± SD	± 1.0	± 3.48	± 4.77	± 7.74

TABLE 2

SUMMARY OF MORPHOMETRIC DATA

Air Space Neutrophils/s Rabbit,* × 10⁸	Total Lung Wt, g/rabbit	Air Space Neutrophils/g Lung × 10⁴	Neutrophils Washed Out, %
9.57	11.8	6.11	42.2
± 9.31	± 4.07	± 3.6	± 10.4

Values are mean ± SD.
* n = 6.

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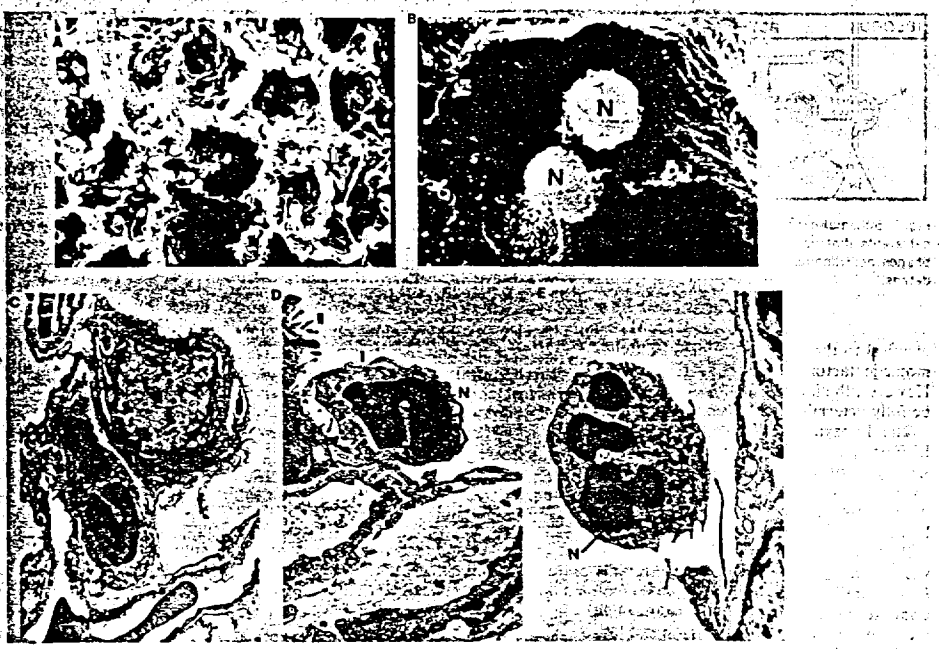

Fig. 4. Anatomy of neutrophil migration into dogs' lungs after repetitive lung washes. A. Scanning electron micrograph. Massive influx of neutrophils into lungs after two previous lavages showing neutrophils in alveoli that are entangled in epithelial cellular debris (Original magnification: x 1300). B. Scanning electron micrograph. Type II epithelial cell with a prominent cell attached and one released in the air space (N) (Original magnification: x 5000). C. Neutrophils in interstitium between capillaries and a Type II cell (II) (Original magnification: x 11,000). D. Neutrophil (N) outside basement membrane covered by a Type I Cell (I) adjacent to a Type II cell (II) (Original magnification: x 11,000). E. Neutrophil (N) in alveolus with epithelial debris (Original magnification: x 15,000).

in which: S_B = the blood neutrophil specific activity, S_A = the alveolar neutrophil specific activity, t_1 = 5 h, t_3 = 9 h. This relationship makes no assumption of linearity of the rate of change of blood specific activity. Turnover time (t_t) of the alveolar neutrophil pool was determined by the relationship:

$$\frac{1}{k} = t_t$$

The k was 0.38 − 0.41 per h and t_t was 2.63 h. The circulating neutrophil pool was 45% of the total blood neutrophil pool.

These experiments are the first quantitative direct estimates of the turnover of neutrophils in an extravascular pool. The results have importance to several areas of investigation. Bierman and colleagues suggested that the lung was a significant site of egress for neutrophils (27, 29). This hypothesis can be examined in the following manner. The average basal neutrophil concentration of the blood of the rabbits was 6.2 × 10^6 cells/ ml with 50% neutrophils. If the rabbits had an average blood volume of about 57 ml/kg (32), and the circulating neutrophil pool represented 45% of the total blood neutrophil pool, then the total blood neutrophil pool was about 3.9 × 10^8 cells per kg. Because the average half-life of the blood neutrophil in this study was 4.5 h, the average turnover time was 6.48 h or 3.7 turnovers per day. Then, about 1.44 × 10^9 blood neutrophils turn over per kg of rabbit each day.

A similar calculation can be carried out for neutrophils in air spaces. The pool size of alveolar neutrophils is 6.11 × 10^4/g lung (S.D. = 3.6) (13). There were 4.94 g of lung per kg weight of rabbit (13) and a turnover time of 2.63 h, or 9.1 turnovers per day. Therefore 2.75 × 10^6 neutrophils turn over per kg of rabbit in the lungs per day and 0.19% of the total neutrophil turnover of the blood occurs in the lungs, so Bierman's hypothesis (5, 7-9) is clearly untenable.

By What Route do Neutrophils Enter Alveoli after the Stimulus of Lung Lavage?

Kazmierowski and colleagues (33) showed that lung washes cause chemotactic factors to be released into the lungs and neutrophils to migrate into the alveoli.

In this study, repetitive lung washes were used to attract large numbers of neutrophils into the airways in order to identify the site of influx of alveolar neutrophils (34). The combined use of scanning and transmission electron microscopy made it possible to visualize the migration of neutrophils from the capillaries, through the interstitial spaces, and through the epithelium into the alveoli.

The use of the scanning electron microscope (SEM) facilitated identification of areas of the lungs that were undergoing massive infiltration with neutrophils (figure 4A). High-power SEM views of the alveoli in the areas of influx of neutrophils demonstrated that numerous neutrophils were attached to and migrating out from around the periphery of the Type II cells (figure 4B). Transmission electron micrographs in these areas also demonstrated the frequent occurrence of neutrophils in close association with Type II cells. Transmission electron micrographs of sections (60 nm thick) cut from the region shown revealed neutrophils in the capillaries (not shown), in the interstitium (figures 4C and D), and in the air spaces (figure 4E). The photomicrographs show neutrophils progressively closer to the alveolar space. A neutrophil in the interstitium adjacent to the capillary and in close proximity to a Type II cell is seen in figure 4C. The association of neutrophils adjacent to Type II cells was a very common occurrence in the region where cells were observed in the air spaces. The neutrophil in figure 4D is seen completely

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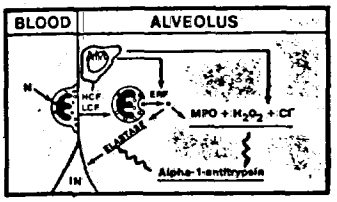

Fig. 7. Schematic diagram of the putative chemical events that occur in alveoli, relating macrophages, neutrophils, and lung tissues. See text for details.

identical to the high molecular weight chemotactic factor described by others (35). However, the identity of this factor cannot be fully determined until it is isolated.

The lysozyme-releasing activity of the ERF was partially destroyed and reduced in size by trypsin. The elastase-releasing activity and lysozyme-releasing activity were destroyed by chymotrypsin but unaffected by neutrophil elastase.

Because it has been shown that more alveolar macrophages (44) and neutrophils (45) are recoverable from lung washes of cigarette smokers, and because neutrophils are found in alveoli of normal animals (13), the introduction of these macrophage factors may cause the release of elastases and promote the degradation of lung elastin in smokers.

The HMW factor caused a 30% increase in release of elastase from neutrophils, compared with untreated control subjects. Individuals who are homozygotes for alpha-1-antitrypsin deficiency (PiZ) have about 15% of normal blood concentrations of alpha-1-antitrypsin and almost all of them develop emphysema (1). Individuals who are heterozygotes for alpha-1-antitrypsin deficiency have about 50% of normal blood levels, and it has been difficult to determine whether they develop emphysema as a result of this lesser deficiency. Therefore 30% differences in elastase released into the lung may be a critical determinant in the development of emphysema.

The release of myeloperoxidase by the ERF also may be important in the genesis of emphysema. Matheson and colleagues (46) showed that myeloperoxidase inactivates alpha-1-antitrypsin in the presence of halide ions and hydrogen peroxide, and both resting and activated lung macrophages secrete hydrogen peroxide into their immediate environment (47, 48). The HMW factor in the area of the macrophage may initiate the release of the myeloperoxidase, which could then destroy alpha-1-antitrypsin. Therefore the HMW factor may cause the release of neutrophil elastase and an agent that destroys its major inhibitor in the lungs. These phenomena, diagrammed in figure 7, may alter the alveolar environment to permit the lung destruction seen in emphysema.

Flux of Neutrophil Elastase Through the Lungs

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Acknowledgment

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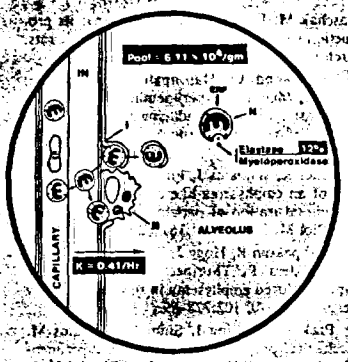

Fig. 5. Schematic diagram of the route that neutrophils take to enter lungs and quantitative aspects of the entrance of neutrophils into the normal lungs of rabbits. See text for details.

extruded from the interstitium but still covered by the Type I epithelial cell. The neutrophil in the air space, with remnants of the epithelial cells seen attached to the cell and breaking away from the alveolar wall, is seen in figure 4E.

In summary, the influx of neutrophils into the alveoli was stimulated by lung lavage (figure 5). Electron micrographic study of these stimulated lungs demonstrated that neutrophils migrate from the capillaries into the interstitium and then migrate between Type II and Type I cells into the alveolar space. The actual migration into the alveolar spaces is effected by breaching the alveolar cell basement membrane and lifting off the alveolar Type I cell membrane. Neutrophils carry fragments of Type I cell membranes into alveoli when they migrate into the alveolar space.

Control of Neutrophil Movement into Lungs

In order to study neutrophils in normal animals, it is necessary to determine what physiologic and chemical forces control neutrophil movement and neutrophil granule deposition in the lungs. As noted above, the importance of chemotactic attraction of neutrophils to the lungs was recently highlighted in studies by Kazmierowski and colleagues (33), who found that repetitive lavage of monkey lungs resulted in a change from macrophages to neutrophils as the predominant cell in the lavage fluid. In addition, they described two chemotactic factors with estimated mass ratios (Mr) of 5,000 and 15,000 in the lavage fluid (33). One was immunologically related to C5 and the other was produced by the alveolar macrophages. Chemotactic factors from human alveolar macrophages were subsequently described by Merrill *et al* (35) and Hunninghake *et al* (36), who found that human macrophages secrete 2 chemotactic factors. The estimated Mr of the smaller factor was between 400 and 600 (36) and Mr of the larger one was about 10,000 (35). The latter is partially destroyed by trypsin (35) and is therefore probably a peptide, while the former may be related to an arachidonic acid metabolite (36).

Because normal macrophages seem to secrete these factors (36, 37) and smokers' macrophages secrete more of these factors, it is perhaps not surprising that smokers have more neutrophils in their lung washes than nonsmokers (12). One might suggest that if the lung needs rapid access to neutrophils in case the macrophagic defense system is overwhelmed by microbes, it would be advantageous to have the mechanism for generating chemotactic agents in place and functioning at a low level and ready to respond quickly to an overwhelming bacterial invader. This adaptive response may explain why the low level of neutrophil traffic occurs in the normal lungs. If, indeed, neutrophil elastase causes emphysema in persons who lack functional alpha-1-antitrypsin in the lungs, then either this low level of neutrophil traffic supplies the necessary elastase to effect the lung changes over 30 to 50 years or the elastase is supplied by acute bursts of neutrophil traffic through the lungs such as may occur, for example, during respiratory infections. There is currently no data to suggest that such bursts occur in the lungs of otherwise normal persons. The topic of chemotactic factors in lungs is addressed more fully by Dr. Reynolds in this supplement.

Fate of Neutrophils in the Normal Lungs

Little is known of the fate of the neutrophils in the normal lungs. The following possibilities exist: (*1*) clearance up the airway, (*2*) return to the circulation, (*3*) degranulation, (*4*) phagocytosis by macrophages, (*5*) lysis and clearance of components, (*6*) clearance into the lymph, and (*7*) some combination of the above possibilities.

Very little data currently exist to implicate one route or another. In previous studies, we showed that although one could find neutrophils in macrophages of different species (figure 6), this seemed to be a rare occurrence. More neutrophil phagocytosis by macrophages may occur in abnormal conditions (38); however, when rabbit neutrophils were added to autologous rabbit alveolar macrophages *in vitro*, very little uptake was seen (13). Campbell and colleagues have determined that alveolar macrophages have surface receptors for human neutrophil elastase (39). This phenomenon may account for some of the clearance of neutrophil elastase in the lungs.

Because only those mechanisms that might result in the introduction of elastase into the lung interstitium or into the alveolus are relevant as a source of enzyme which may produce emphysema, only possibilities 3, 5, and 7 are important for this discussion. Some data are available regarding neutrophil degranulation. It is well known that neutrophils degranulate in the presence of certain chemotactic factors such as C5A when cytochalasin B is added (40) or in the absence of cytochalasin B when the neutrophils adhere to membranes (41). Others have demonstrated neutrophil degranulation by chemotactic factors from human alveolar macrophages treated with cytochalasin B; however, they have found that the low molecular weight factor causes only degranulation of specific granules that do not contain elastase or myeloperoxidase.

We have determined that human alveolar macrophages secrete an enzyme releasing factor (ERF) capable of causing release of myeloperoxidase, lysozyme, and elastase from cytochalasin B-treated human neutrophils and release of elastase from neutrophils associated with millipore membranes in the absence of cytochalasin B (42). Based on the calculation of Kruze *et al* (43), one can estimate that the ERF released about 12.5% of the elastase in the neutrophils. It seems likely that the factor is

Fig. 6. Degranulated neutrophil that appears to be in an alveolar macrophage in the lung wash of a rabbit (Original magnification: x 1,000).

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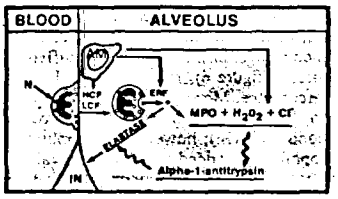

Fig. 7. Schematic diagram of the putative chemical events that occur in alveoli, relating macrophages, neutrophils, and lung tissues. See text for details.

identical to the high molecular weight chemotactic factor described by others (35). However, the identity of this factor cannot be fully determined until it is isolated.

The lysozyme-releasing activity of the ERF was partially destroyed and reduced in size by trypsin. The elastase-releasing activity and lysozyme-releasing activity were destroyed by chymotrypsin but unaffected by neutrophil elastase.

Because it has been shown that more alveolar macrophages (44) and neutrophils (45) are recoverable from lung washes of cigarette smokers, and because neutrophils are found in alveoli of normal animals (13), the introduction of these macrophage factors may cause the release of elastases and promote the degradation of lung elastin in smokers.

The HMW factor caused a 30% increase in release of elastase from neutrophils, compared with untreated control subjects. Individuals who are homozygotes for alpha-1-antitrypsin deficiency (PiZ) have about 15% of normal blood concentrations of alpha-1-antitrypsin and almost all of them develop emphysema (1). Individuals who are heterozygotes for alpha-1-antitrypsin deficiency have about 50% of normal blood levels, and it has been difficult to determine whether they develop emphysema as a result of this lesser deficiency. Therefore 30% differences in elastase released into the lung may be a critical determinant in the development of emphysema.

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Neutrophil Enzymes in the Lung: Regulation of Neutrophil Elastase[1–3]

STEWART A. LONKY and JOHN McCARREN

Introduction

In 1963, Laurell and Erickson (1) recognized a higher than expected incidence of lung disease in patients with an inherited deficiency of alpha 1 protease inhibitor (A1Pi). Since that time, Janoff and Scherer (2) have demonstrated that the granules of human neutrophils contain proteolytic enzymes with activity at neutral pH, whereas a number of other investigators have shown that the intratracheal administration (3, 4) of neutrophil elastase (NE) induces experimental emphysema in laboratory animals. These data have prompted the notion that a relative excess of NE in the lung leads to elastic tissue disruption and pulmonary emphysema. The fact that the majority of patients with emphysema have normal serum levels of A1Pi suggests that if such a protease pathogenesis theory is correct, periods of imbalance between elastolytic enzymes and elastolytic inhibitors must exist in the lung. Accordingly, a number of laboratories have sought to identify factors that either diminish lung antiprotease activity or increase NE activity in the lung. In this report we shall address our attention to the data regarding factors that increase NE activity either intracellularly or after the enzyme has been released from neutrophil granules.

[1] From the Department of Medicine, University of California, San Diego, and Division of Pulmonary and Critical Care Medicine, Veterans Administration Medical Center, San Diego, California.

[2] Presented in part at the Annual Meeting of the American Thoracic Society, Detroit, Michigan, May 10, 1981.

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The Intracellular Regulation of Neutrophil Elastase

Synthesis of the Enzyme

In the mature neutrophil, elastolytic enzyme activity is found in the azurophilic granules (5–7). These granules are formed only during the promyelocyte stage of neutrophil development (8), and are subsequently distributed to daughter cells following cellular mitosis. Mitotic divisions cease after the myelocyte stage, and further cell maturation is accompanied by a decrease in cellular organelles essential for protein synthesis. Specifically, the nuclear chromatin becomes coarsely clumped, nucleoli disappear, and there is a dramatic reduction in free ribosomes and rough endoplasmic reticula (8). These findings suggest that the elastase content of the mature neutrophil is determined during the early stages of granulopoiesis. However, Granelli-Piperno and associates (9) have shown that more than 60% of mature human neutrophils isolated from peripheral blood retain the capacity to synthesize RNA and other proteins. In addition, both neutrophil biosynthetic activity and the release of newly synthesized macromolecules are influenced by a variety of chemicals.

Clark and coworkers (10), using cytochemical techniques, found evidence for the existence of elastase-like enzymes within the nuclear envelope, endoplasmic reticulum, golgi complex, and some granules of circulating neutrophils. Thus it appears that the circulating neutrophil is capable of NE synthesis. Whether factors that increase NE activity act at the level of neutrophil precursors or on mature neutrophils is yet to be determined.

Clinical Correlates of Increased Neutrophil Elastase

In an attempt to establish a relationship between protease-antiprotease imbalance and clinical disease, a number of investigators have measured the levels of NE in patients with chronic obstructive pulmonary disease (COPD), and compared these with levels measured in normal subjects. Goldston and colleagues (11) reported there are increased degrees of neutrophil elastase-like esterolytic activity in patients with COPD and a variety of Pi phenotypes. Lam and coworkers (12) demonstrated that higher than normal levels of NE constitute a significant risk factor for the development of COPD in PiMZ patients, and Rodriguez and associates (13) found that NE levels are increased in COPD patients with PiMM phenotype. Although Kramps and coworkers (14) failed to find any correlation between NE levels and the presence of lung disease in PiZZ patients, these investigators did note that PiMM patients with COPD had higher than normal levels of NE. These studies suggest that high levels of NE represent a potential risk factor for the development of COPD, and that other factors, such as the status of lung antiprotease activity, also play an important role in determining the presence or absence of clinical disease.

Attempts to correlate the level of NE in peripheral blood with disease severity have not been uniformly successful. Although Kidokoro and coworkers (15) found more lung hyperinflation and more severe impairment of carbon monoxide diffusing capacity in PiZZ patients with higher NE levels, Rodriguez and associates (13) failed to find any such correlation in COPD patients with the PiMM or PiMZ phenotype. Additional, well controlled studies are necessary before this issue can be resolved, but it is reasonable to believe that NE levels are one of the variables that dictate the degree of connective tissue disruption in COPD.

Relatively little information is available regarding the factors that regulate the level of NE in circulating or lung neutrophils. It has been demonstrated (13, 15) that these levels are relatively stable over a period of 6 months to 1 yr in patients with uncomplicated disease, and it appears that these NE levels are not dependent on age, sex, or Pi type (11, 14). Although it has been suggested that intracellular NE levels might be genetically determined (16), there are preliminary data indicating that both transient and more sustained increases in NE activity can be acquired. Cigarette smokers have increased levels of NE when compared with matched control subjects (12), and both patients (13) and laboratory animals (17) with pneumococcal pneumonia have transient elevations in NE activity.

Neutrophil Elastase and Pneumococcal Pneumonia: Protease Antiprotease Homeostasis

Design of the study. The longstanding association between infection and COPD prompted us to investigate the relationship between NE levels and pulmonary infection. Pulmonary infection with the Type III pneumococcus was selected as an experimental model because infection with this organism induces a brisk-neutrophil response in the lung. Furthermore, nonlethal lobar pneumonia can be induced in dogs by intratracheal inoculation of this organism suspended in starch broth (18, 19). Conditioned dogs were inoculated with either normal saline (control dogs) or Type III pneumococcus (pneumonia dogs), and the inoculum was directed to the right lower lobe under fluoroscopic guidance. Serial roentgenographs, CBCs, and temperature measurements confirmed that a lobar pneumonia was induced in 11 of 14 dogs, and cultures of bronchial and lung aspirate confirmed that the pneumococcus was the bacterial agent. No control animal developed pneumonia. Peripheral blood granulocytes were collected from 50 ml of blood (17), and elastase-like esterase activity against N-t-Boc alanine-p-nitrophenyl ester (NBA) was measured (20). In addition, peripheral blood antiprotease activity was determined (21). Bronchoalveolar lavage was performed via a fiberoptic bronchoscope wedged in the uninoculated left lower lobe, and bronchoalveolar lavage fluid (BALF) and cells were collected.

Time course and magnitude of NE response. Within 24 hours, the 9 dogs surviving pneumonia induction demonstrated a statistically significant increase in circulating neutrophils (6.5 ± 0.9 to 14.9 ± 2.1 × 10^3 cell m/ml), and an increase in the percentage of circulating immature neutrophils from 4% to 24%. Although the mean circulating neutrophil count increased somewhat in 5 control animals, this increase was not statistically significant. Within 24 hours of pneumococcal inoculation, infected dogs showed a 40 to 60% increase in NBA esterase levels compared with preinfection levels, and this increase in circulating NBA esterase activity persisted for 48 h (figure 1). A similar increase in NBA esterase activity was found in neutrophils derived from the BALF of infected dogs, indicating that the lung and its connective tissue substrate is exposed to this increased enzymatic potential. Preliminary data from humans with acute bacterial pneumonia support this finding in dogs, revealing transient, statistically significant increases in circulating NE levels during active infection (13).

It is of interest to note that uninfected animals also experienced an increase in circulating neutrophil elastase-like esterase activity, but that this increase occurred more slowly, reaching statistical significance only on the fourth post-infection day (figure 1). Of these 5 control animals, 3 had bronchoalveolar lavage performed daily, whereas the other 2 had lavage performed only once (on the day of inoculation).

A further analysis of data from these animals revealed that the increase in NBA esterase activity shown in figure 1 for con-

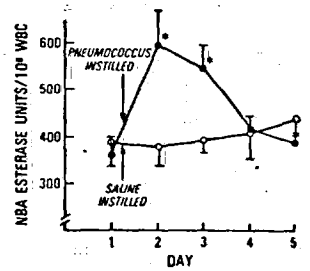

Fig. 1. Changes in mean circulating NBA esterase activity in 9 dogs intratracheal pneumococcus (*closed circles*) and in 5 dogs receiving intratracheal saline (*open circles*). Asterisks signify that NBA esterase levels are higher in pneumonia dogs with $p < 0.02$ (Student's *t* test).

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trol dogs can be attributed to the lavage process because unlavaged control subjects had only a 2 to 4% increase in NBA esterase activity, whereas lavaged animals experienced a 14.5% increase (17). No significant increase in the circulating neutrophil count was detected in the 3 control dogs that underwent bronchoalveolar lavage, but each of these 3 animals experienced a brisk influx of neutrophils to the lung. Studies in monkeys and in a large number of dogs have verified that either bronchoscopy or bronchoalveolar lavage is associated with a pulmonary neutrophil response (22). These studies also reported an increase in peripheral blood neutrophils and an increase in immature band forms following bronchoscopy or lavage. Therefore, in addition to pneumococcal pneumonia, bronchoscopy and repeated bronchoalveolar lavage can expose the lungs to increased levels of NE.

Time course and magnitude of antiprotease response. The data described thus far suggest that pulmonary infection can result in an increased risk for elastolysis. The fact that pneumococcal pneumonia is not usually followed by impaired lung elasticity prompted us to measure the protease inhibitory activity in the peripheral blood and lungs of infected and noninfected animals. We found that the serum trypsin inhibitory capacity (TIC) and the serum NBA esterase inhibitory activity (EIC) increased 15% in dogs with pneumonia, and remained elevated for 48 h (figure 2). Furthermore, we found a striking increase in BALF NBA esterase inhibitory activity following infection, with the mean inhibitory activity increasing from 776 ± 200 to 1400 ± 190 NBA units inhibited/mg lavage protein (mean ± SE).

Subsequent studies have shown that this increase in lavage EIC is due to an increase in A1Pi. Thus it appears that the lung is protected from proteolytic attack during pneumococcal lung infection by a simultaneous increase in peripheral blood and lung protease inhibitory activities. Control animals did not demonstrate any statistically significant changes in either peripheral blood or lung lavage antiprotease activity, even when control animals undergoing bronchoalveolar lavage were considered separately. These data suggest that protease and antiprotease activities following bronchoscopy and bronchoalveolar lavage may be modulated by independent factors.

Extracellular Regulation of Neutrophil Elastase

Once the neutrophil has migrated to the lung, a variety of stimuli can trigger the extracellular release of granular contents. Models for the mechanism of extracellular granule release have been reviewed elsewhere (23), and will not be reconsidered here. For NE, the available data indicate that it is released from neutrophil granules as an active enzyme, free to act on any of its substrates. Whether NE actually attacks a particular substrate, however, depends on the balance struck between factors that either enhance or diminish NE activity. Such factors include the location of enzyme release, the presence or absence of enzyme inhibitors, and the presence of various molecules that can enhance NE activity.

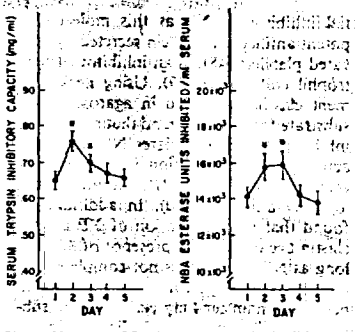

Fig. 2. Mean ± SE of serum trypsin inhibitory capacity (*left*) and serum NBA esterase inhibitory capacity (*right*) in 9 dogs inoculated with pneumococcus. All animals inoculated on Day 1. Asterisks signify levels significantly greater than preinfection levels with $p < 0.05$ (Student's *t* test).

Location of Enzyme Release

In the lung, NE can be released into any of the 3 major compartments: vascular, interstitial, or bronchoalveolar. Potential substrates for this enzyme exist in each compartment because NE is a proteolytic as well as an elastolytic enzyme. NE will attack a variety of proteins, including complement (24), components of the clotting system (25), immunoglobulins (25), kinins (25), collagen (26), basement membranes (27, 28), and elastin (3, 29). It is, however, the action of NE against lung elastic tissue and the induction of an emphysema-like lesion (3, 5) that has been most extensively studied, and which will be reviewed here.

Because elastin is found in the lung interstitium, NE release into this compartment would be expected to result in the greatest amount of elastic tissue damage. Although such interstitial release of NE has not been directly measured, there is evidence that such release takes place. Wright and Gallin (30) have shown that the chemotaxis of PMNs is associated with the leakage of various enzymes, and the movement of PMNs through the interstitial space can be expected to result in small amounts of NE release. In addition, a variety of inflammatory reactions in the lung are associated with the interstitial accumulation of PMNs. Some of these reactions are due to agents that are capable of causing PMN degranulation (23), and NE release is inevitable under these circumstances.

Because both the vascular and bronchoalveolar compartments contain protease inhibitors, NE released into these compartments is subject to inactivation. As the vascular compartment contains a greater amount of inhibitory protein, one would expect that lung elastolytic damage would be less if NE were introduced via this route. Animal studies have borne out this hypothesis, demonstrating that although experimental emphysema can be induced by the intravascular administration of elastolytic enzymes (31), enzyme administered intratracheally is more potent against lung elastin (32).

NE released into the bronchoalveolar compartment is subject to the effects of various inhaled gases, aspirated material, and airborne particulate matter. Any of these factors can alter the physical-chemical environment in which the enzyme will act, resulting in a change in enzyme activity. To date, there have been few studies directed at measuring such changes in NE activity *in vivo* but we can predict some expected effects by considering the available *in vitro* data. The elastolytic activity of NE is decreased when the pH is decreased below 7.4 and approaches zero at pH = 5.0 (33). It is intriguing to hypothesize that the inhalation of gases such as ozone, oxides of nitrogen, or chlorine, which can diminish the pH of bronchial secretions, might decrease NE activity. In addition, the aspiration of acid stomach contents might inhibit the action of free bronchoalveolar NE. Conversely, the alkalinization of bronchial secretions might result in enzyme stimulation because NE reaches peak elastolytic activity at pH 8.8 (33). Changes in the ionic strength of bronchial secretions, induced by the transudation of fluid or the inhalation of various solutions, also might affect NE activity. Increases in ionic strength can result in NE stimulation against both elastin (34) and synthetic substrates (35), whereas decreases in ionic strength can diminish NE elastolytic activity.

Presence of Protease Inhibitors

In each of the lung compartments are protease inhibitors that can neutralize NE activity. As previously stated, the major serum and bronchoalveolar fluid inhibitor of NE is A1Pi (36). The mechanism of action of this protease inhibitor has been described (37), and the functional regulation of A1Pi is the subject of another report in this symposium. A1Pi is made predominantly in the liver (38). More recent data have demonstrated that the alveolar macrophage is also capable of A1Pi synthesis (39), although it is likely that macrophage-derived A1Pi constitutes only a fraction of BALF A1Pi. In the serum, A1Pi is an acute phase reactant with increases in levels occurring in response to lobar pneumonia, pregnancy, birth control pills, malignant tumors, severe burns, and typhoid vaccine (38). In the bronchoalveolar compartment, A1Pi levels are increased following infection with the pneumococcus (17), and in the

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bronchoalveolar lavage fluid of smokers (40). Other factors that might increase bronchoalveolar levels of this protease inhibitor are still to be identified. Because this compartment is exposed to inhaled and aspirated material, it is likely that the level or activity of bronchoalveolar A1Pi is more susceptible to change than serum levels or activity.

In addition to A1Pi, both the vascular and bronchoalveolar compartments contain alpha 2 macroglobulin (α2M), a large (MW = 725,000) protein that can inhibit NE elastolytic activity (41). Whereas A1Pi completely inhibits NE activity, experimental data have shown that NE activity against elastin is incompletely inhibited by α2M (41). The residual activity of α2M-NE complexes against elastin may play an important role in patients who are genetically or functionally deficient in A1Pi (41, 42). Two other NE inhibitors have been identified in the bronchoalveolar compartment, neither of which has been fully characterized. The first of these is a low molecular weight (MW = 14,000) acid-stable inhibitor found in purulent sputum (43) and bronchoalveolar lavage fluid (44). The second bronchoalveolar inhibitor is a high molecular weight (MW = 80,000) inhibitor found in bronchoalveolar lavage fluid of dogs and humans (45). The role of these inhibitors *in vivo* is yet to be ascertained.

Factors That Stimulate NE Activity

Substrate considerations. Elastin is a cross-linked structural protein containing an unusually high percentage of nonpolar amino acids (46). These qualities render elastin insoluble in aqueous systems. Because of this, multiple synthetic substrates have been developed that allow for the rapid spectrophotometric determination of elastase activity. The most widely used of these substrates are N-t-Boc-L-alanine-p-nitrophenyl ester (NBA) and succinyl-trialanine-p nitroanilide (Suc-$(Ala)_3$-NA), each of which has been used as substrate for pancreatic elastase or NE (18, 47). It must be remembered, however, that these synthetic substrates measure the esterolytic activity of NE, and although this esterolytic activity parallels the elastolytic activity of the enzyme, factors that influence the esterase activity of NE may have little or no effect on the elastolytic activity of the enzyme, and vice versa. This fact is well demonstrated by reviewing the effects of α2M on NE activity. Whereas NE elastolytic activity is almost completely abolished by α2M, the activity of NE against Suc-$(Ala)_3$-NA is markedly increased. Because it is elastin that is the substrate in lung parenchyma, we have focused our attention on factors that stimulate NE activity against either lung elastin or elastin purified from lung tissue or bovine ligamentum nuchae.

Stimulation of NE by platelet factor 4. We are initially interested in PF_4 as a potential inhibitor of NE as this molecule, a potent antiheparin protein secreted by activated platelets (48), is an inhibitor of neutrophil collagenase (49). Using neck ligament elastin suspended in agarose as the substrate for NE, we found that rather than inhibit NE, PF_4 stimulates NE elastolytic activity. This stimulation is seen at concentrations of PF_4 and NE that are physiologically attainable (50). In addition, we found that this stimulation of NE against elastin occurred in the presence of A1Pi as long as NE activity was not completely inhibited. Studies performed using radiolabeled (51) hamster lung elastin as the substrate confirmed these findings, demonstrating a 50% increase in NE activity when the molar ratio of PF_4:NE was 4:1, and a doubling of NE activity when the ratio was 10:1 (figure 3). When a synthetic NE substrate such as NBA was used, we found no enhancement of NE esterolytic activity (figure 3). The proteolytic activity of NE against either casein or hemoglobin is only slightly increased at high molar ratio of PF_4:NE (figure 3). Thus the ability of PF_4 to stimulate NE is relatively specific for NE elastolytic activity.

To test whether PF_4 stimulated NE activity against lung elastin in situ, hamster lungs were inflated with solutions of buffer alone (phosphate buffered saline pH 7.4; PBS), human NE, PF_4 alone, or PF_4 and NE mixed at a molar ratio of PF_4:NE = 4:1. After a 2-hour incubation at 37° C, the lungs inflated with NE alone showed minimal alveolar hyperinflation. Pressure-volume studies revealed that the compliance of these "low dose" NE lungs was not different from the compliance of control lungs. The addition of PF_4 to the same amount of NE resulted in a more severe alveolar injury (figure 4) and a shift of the lung pressure-volume curve to the left (figure 5). Measurements of lung connective tissue content revealed that a significant decrease in measurable lung elastin occurred only in the animals treated with NE plus PF_4, and that collagen content was unaffected (51). Thus, PF_4 is capable of stimulating NE against lung elastin in situ, and such stimulation may result in connective tissue destruction by levels of NE that otherwise would be ineffective.

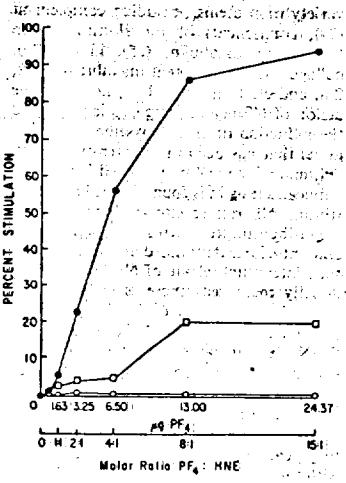

Fig. 3. Stimulation of human neutrophil elastase (HNE) by PF_4 against ^{3}H-lung elastin (*closed circles*), ^{125}I-casein (*open squares*), and N-t-boc-alanine-p-nitrophenyl ester (NBA; *open circles*). All experiments done with 5 μg HNE. 5 μg resulted in release of 1.9×10^3 cpm from ^{3}H elastin, and 1.2×10^3 cpm from ^{125}I casein. Incubations at 37° C for 3 h.

PF_4 as a model stimulant of NE activity; effects of other lysine-rich ligands. PF_4 is a basic protein at physiologic pH, and a study of the amino acid sequence of this protein reveals that its cationic nature is the result of a lysine-rich region near the carboxy-terminus of this 70 amino acid molecule

61 65

(. . . Lys-Lys-Ile-Ile-Lys-Lys . . .) (52). We have found that upon mixing with NE, PF_4 is rapidly cleaved into at least 3 fragments (51). However, PF_4 which has been incubated with NE for 1 hour, still retains NE stimulatory activity, suggesting that one or more of the PF_4 fragments are capable of stimulating NE. Preliminary data have shown that some of the PF_4 fragments contain the lysine-rich carboxy terminal region and remain cationic. These data prompted us to study the effect of other lysine-rich molecules on NE activity.

To date we have studied various poly-L-lysines and histones that are relatively lysine-rich (histone V and histone IIIs; Sigma, St. Louis, MO). We found that poly-L-lysines with a polymer length greater than 4 lysines are capable of stimulating NE activity (table I), and that histone IIIs is also capable of such stimulation. This property is not found in other basic molecules because poly-L-arginine, α chymotrypsin, and trypsin fail to stimulate NE activity (table I).

Previous investigators have shown that elastin is slightly anionic in nature (53), and Gertler (54) has shown that basic ligands such as poly-L-lysine, poly-L-arginine, and pancreatic elastase bind to elastin in a non-specific, electrostatic manner. We expect that PF_4 and other lysine-rich ligands also bind to elastin electrostatically, and that this substrate binding plays an important role in NE stimulation. This theory is in disagreement with experimental observations for pancreatic elastase, which show that pancreatic elastase activity against elastin is wholly governed by the presence of charge complementarity between enzyme and substrate (55). However, for NE, electrostatic binding of enzyme to substrate is likely to be less important than for pancreatic elastase. Evidence that NE intereacts

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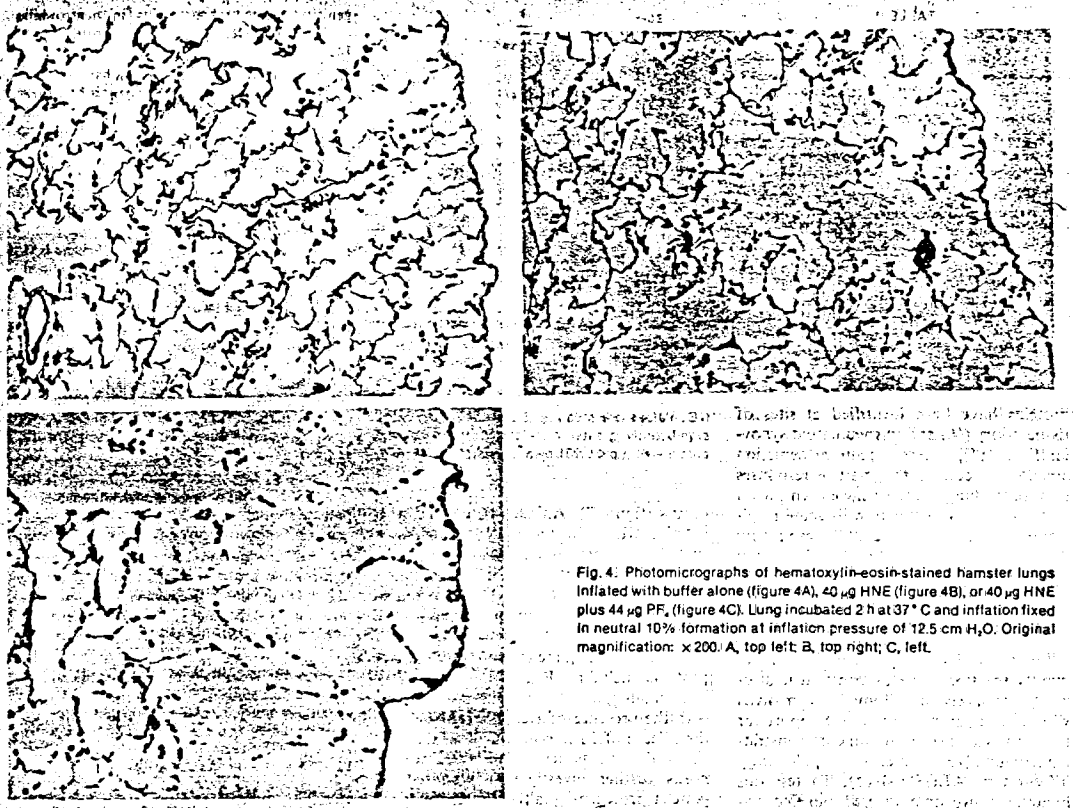

Fig. 4. Photomicrographs of hematoxylin-eosin-stained hamster lungs inflated with buffer alone (figure 4A), 40 μg HNE (figure 4B), or 40 μg HNE plus 44 μg PF_4 (figure 4C). Lung incubated 2 h at 37° C and inflation fixed in neutral 10% formation at inflation pressure of 12.5 cm H_2O. Original magnification: ×200. A, top left; B, top right; C, left.

with elastin via nonelectrostatic mechanisms comes from work by Boudier and coworkers (34) and Liestienne and Bieth (35), who have shown that NE elastolytic and esterolytic activity are increased in the presence of high ionic strength, a condition that rapidly inactivates the pancreatic enzyme (25, 33).

Support for the hypothesis that PF_4 stimulates NE by attaching to the substrate (substrate directed stimulation), rather than by a direct action of PF_4 on NE, comes from experiments in which PF_4 was preinfused into elastin-agarose plates and followed by the addition of NE (figure 6). Stimulation of NE results, and the degree of stimulation is somewhat greater than that seen when PF_4 and NE are mixed before addition to the elastin plate wells.

From these data we have theorized that PF_4 released into the lung, even in the absence of concomitant NE release, can be adsorbed onto elastin, resulting in a substrate that is more susceptible to attack by NE at a later time.

Platelets and PF_4 in the lung; the "inflammatory" platelet. The data outlined above take on increased significance when one considers the fact that platelets are a normal constituent of circulating blood, and that activated platelets can release PF_4 endogenously (56). Recent evidence has pointed to an additional role for platelets, that being a role in the inflammatory reaction. Several agents that initiate inflammatory reactions, such as urate crystals (57), immune aggregates (58), and bacterial lipopoly saccharides (59), can stimulate platelets and result in the release of platelet contents. In addition, platelets and platelet

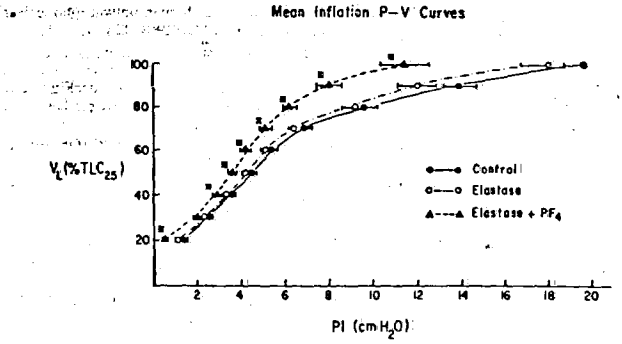

Fig. 5. Mean inflation PV curves for animals receiving buffer alone (●), 40 μg HNE (○), or 40 μg HNE plus 44 μg PF_4 (▲). Asterisks indicate data points significantly different from control values by analysis of variance. Mean deflation P-V curve (not shown) reveals similar results, with HNE + PF_4 curve significantly more compliant at lung volumes (V_L) from 50% to 100% of total lung capacity (TLC_{25}).

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TABLE 1
RELATIVE ELASTOLYTIC ACTIVITY OF NEUTROPHIL ELASTASE[a,b]

Ligand Tested	Molar Ratio Cationic Ligand:NE 2:1	4:1	10:1
Poly-L-lysine (30,000)	2.20	2.20	2.20
Poly-L-lysine (13,000)	1.70	1.90	2.0
PF_4	1.50	1.75	1.75
Histone IIIs	1.18	1.35	1.55
Poly-L-lysine (3,400)	1.30	1.40	1.45
Lysine (8-12)	1.05	1.10	1.25
Tetralysine	1.00	0.95	0.95
ϵ chymotrypsin	1.00	0.90	0.90
Poly-L-arginine (13,900)	0.90	0.85	0.80

a. Activity of 5 μg NE alone against ^{3}H-elastin = 1.0.
b. All experiments done using 5 μg NE.

proteins have been identified at sites of tissue injury (60) and in rheumatoid synovial fluid (61), whereas platelet depletion ameliorates certain flammatory responses (56). In the lung, platelets have been shown to accumulate in patients with acute respiratory failure (62) or the adult respiratory distress syndrome (ARDS) (63). Thus, the platelet probably plays an important role in lung inflammation, and we investigated the possibility that lung PF_4 release could occur *in vivo*.

Because PMNs play a role in the inflammatory reaction, we reasoned that neutrophil products such as NE might be involved with a lung platelet response. Support for this notion comes from work demonstrating free lung NE in patients with pulmonary infection or ARDS (63-65). To test this hypothesis, we administered purified human NE intratracheally to rabbits with ^{111}Indium-labeled platelets circulating in their peripheral blood pool (66, 67). We found that within 60 min of NE inoculation, a significant increase in lung platelets occurs (figure 7). Animals inoculated with inactive NE or buffer alone do not show such lung platelet sequestration. In addition, measurement of plasma and bronchoalveolar lavage fluid PF_4 levels by radioimmunoassay revealed that platelets were activated in the NE-inoculated animals. These activated platelets secreted platelet proteins including PF_4. Thus pulmonary inflammatory reactions that are associated with the presence of free NE can result in the release of PF_4. Whether other lung injuries also are associated with PF_4 release needs further investigation because this platelet protein may alter the reactivity of lung elastin, creating an imbalance in the lung protease-antiprotease system.

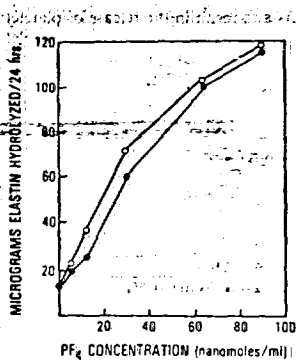

Fig. 6. Stimulation of human NE by PF_4 against elastin in elastin-agar plates. (Alphasin, Elastin Products, St. Louis, MO). Closed circles (●) show results when 16.6 nanomoles of NE preincubated with PF_4 and then added to plate. Open circles (○) show results when PF_4 is preinfused into elastin-agar, and NE added later.

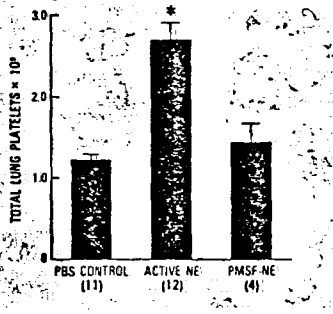

Fig. 7. Bar graph showing lung platelet counts 60 min after intratracheal inoculation of phosphate buffered saline (PBS), human neutrophil elastase (active NE), or human neutrophil elastase inactivated by phenyl methyl sulfonyl fluoride (PMSF-NE). Values are mean ± SE. Asterisk indicates significantly greater platelets in NE-inoculated animals with $p < 0.001$ by analysis of variance.

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Lung Inflammation: Role of Endogenous Chemotactic Factors in Attracting Polymorphonuclear Granulocytes[1-4]

HERBERT Y. REYNOLDS

SUMMARY The inflammatory reaction in the lungs can be considered a mechanism of host defense that augments local alveolar cellular and humoral defense against microorganisms and particulates which challenge the airways. As part of this reaction, the accumulation of blood inflammatory and phagocytic cells, primarily PMNs, and fluid components from plasma may be under control of chemoattractant factors and vasoactive mediators. From the air-side, chemotactic factors originating from alveolar macrophages or through activation of the complement system seem essential in initiating the influx of PMNs into the alveolar space. The kinetics of synthesis and release of chemotactic factors from alveolar macrophages of animals and humans and the status of their immunochemical analysis is the essence of this report. Coupling phagocytosis (afferent function) with its capacity to secrete several kinds of effector molecules (chemotactic factors, complement components, leukotrienes, and platelet activating factor), the alveolar macrophage is considered to have a pivotal role in overall regulation of the inflammatory reaction.

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Introduction

The airways of the respiratory tract are constantly subjected to foreign debris alighting on their surface, which is inhaled with ambient air or admixed with nasooropharyngeal secretions that are aspirated. However, the respiratory tract has an elaborate array of cleansing mechanisms that effectively remove this debris and, under usual circumstances, no harm occurs. These mechanisms are part of a broad umbrella of pulmonary host defenses that protect the health and integrity of the air-exchange surface of the lungs (1).

Counterattacking, therefore, the influx of airborne particles and microbes that enter the conducting airways are formidable anatomic barriers, such as the larynx and the branching structure of the airways, a perpetual ciliary clearance apparatus, locally secreted proteinacious substances containing mucus and immunoglobulins that coat the ciliated epithelium, and a cough mechanism. Further down in the terminal airways and alveoli, phagocytic cells, principally alveolar macrophages, scavenge debris reaching the actual air exchange area.

In sum, these surveillance mechanisms (2) efficiently guard and cleanse the respiratory tract. In addition, other augmenting mechanisms are available and may be operant only at crucial times to aid the usually effective surveillance apparatus. The capacity to make specific antibody in response to a micro-organism that at a future encounter would accelerate its clearance, or prevent attachment to the airway epithelium, is an example. The capacity to generate an appropriate inflammatory response in lung parenchyma is another (3). Thus the mobilization of inflammatory cells and fluid factors from the intravascular compartment into the lung is crucial for effective pulmonary host defenses.

Obviously, full mobilization of the inflammatory reaction leading to pneumonitis and clinical illness occurs infrequently in healthy individuals who are not exposed in an extraordinary way to massive inocula or unusually virulent microbes, but some lesser gradation of the reaction may be needed frequently to combat inhaled and aspirated particulates and microbes that incessantly plumb the depths of the airways. On the other hand, inappropriate or excessive inflammation in the lungs may create pathology that is part of many chronic diseases that affect the lungs. Certain allergic, vasculitic, and autoimmune diseases feature abnormal recruitment of inflammatory cells to the lungs as do a variety of toxic environmental exposures such as cigarette smoke, inhaled asbestos, and silica particles. Accompanying the cellular component of inflammation are its by-products in the form of lysosomal enzymes, oxygen radicals, and a myriad of proteases that can cause destructive injury. Important in the initiation and perpetuation of the inflammatory reaction is the role of chemoattractant factors that help direct and recruit inflammatory cells into extravascular spaces (4). The origin of such factors is multiple and they can arise from complement cascade activation, be synthetic products of cells, or be generated from the debris of inflammation itself that contains a mixture of senescent, injured leaking cells, and plasma protein components.

This report will concentrate on the chemotactic factors that can arise or be generated in the airways and alveoli, and that seem to have relevance in initiating an inflammatory reaction in response to airway challenge. The resulting inflammation is a controlled biologic reaction, we feel (3), and at times it may be a modest, self-limited reaction, perhaps undetectable clinically, but at other times it becomes significantly amplified so that clinical illness is perceived. When a specific microbial organism is the cause, pneumonitis appropriately describes the reaction; when the cause is a noninfectious one, or perhaps is of unknown etiology as is the case for many of the chronic alveolar-interstitial inflammatory diseases, alveolitis is a reasonable term. Where possible, we will emphasize the human situation but recognize the fact that experimental animal studies have been fundamental in dissecting and manipulating the inflammatory response and will remain indispensable for future work.

One final qualifying statement should be made as we embark on a review that will emphasize the role of polymorphonuclear neutrophils (PMNs) in the inflammatory response. We shall ignore the granulomatous-type reaction that prominently includes lymphocytes and transformation of macrophages into epitheloid cells, etc. Some lung research literature indicates that alveolar macrophages liberate factors that influence the accumulation of lymphocytes and that lymphocyte-derived factors affect macrophage motility. Certainly these mechanisms must be considered as well in developing a comprehensive view of the complex cellular mechanisms of lung inflammation.

Baseline—What Inflammatory Cells Are Already in the Airways?

Lung washing techniques that retrieve respiratory cells from the alveolar and distal airway surfaces of animals and humans are the only available means to sample normal lungs in a relatively noninvasive way. Analysis of cells so obtained, now reported by

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many investigators, reveals that the preponderance of cells are alveolar macrophages (<90%), perhaps 4 to 8% are lymphocytes, and 1 to 2% are PMNs. Rarely are eosinophils or basophils detected. These cells, of course, represent the detachable population of cells that are dislodged with infusion and aspiration of saline fluid introduced into the airways. This lavage by no means retrieves all cells present in the lung area sampled, and just how thorough or representative the washing sample is has not been correlated with actual histology in normal humans.

Recent data quantifying PMNs in the lungs of normal rabbits are relevant (5). PMNs constituted about 10% of the cells observed to be free in the alveoli, but only 2.5% of respiratory cells recovered by lung lavage were actually PMNs. It was estimated that lavage effectively removed about 42% of PMNs located in the air space (range of sampling efficiency was 28 to 56%). PMN counts in right and left ventricular blood samples were almost identical so significant trapping of PMNs in the vasculature was not believed to occur. About 6 × 10^4 air space neutrophils per g of lung tissue were calculated. Alveolar macrophages were not observed to phagocytose or to otherwise remove PMNs. Aged or senescent PMNs, however, can be ingested under *in vitro* conditions by macrophages, and this may still be a pathway for cell removal when studied more thoroughly.

To establish basal conditions, it is unclear how many PMNs are naturally present in vascular and capillary areas of normal nonsmokers' lungs as part of the marginated pool of PMNs, or how many are located in interstitial portions of lung parenchyma. Because the lungs contain a large capillary network that serves as an important filter at the interface of the arterial and venous compartments of the circulation, the availability of PMNs within the lung vasculature would seem to be an important feature of host defense. The PMN being an endstage cell with a limited lifespan of a few hours, the mere presence of tissue PMNs in the lungs suggests an active influx and turnover of these cells. In contrast, no other inflammatory cell type seems to be present in normal lungs in a sufficiently high number to make it an important candidate for inclusion in the inflammatory reservoir of the lung. Specifically, eosinophils come to mind. Clearly, eosinophils are important components of allergic lung diseases and various eosinophilic syndromes, but they are not a prominent part of the usual bacterial infection–pneumonitis response. Moreover, as we shall see, the specific chemotactic factors liberated by alveolar macrophages have consistently been weak and negligible stimuli for eosinophils.

Few PMNs have been found in human lung lavage specimens; this paucity was noted in the early studies that used normal subjects (6–9). As illustrated with our group of nonsmoking volunteer subjects (table 1), the differential cell count of respiratory cells recovered with BAL shows that 1.5% of the cells were PMNs (10). For the smoker groups, the percentage increased to 2.4 and 3.7. These increases become more impressive when they are related to the total cell yield and are presented as the total PMN count (figure 1). All nonsmokers had less than 10^6 PMNs per lavage cell total, and 10 of 34 had no evidence of PMNs among their lavage cells. Most marijuana and tobacco smokers had a logarithm greater PMN count than nonsmokers. Values in males and females did not differ. Thus, smokers in general would seem to have a heavier burden of PMNs in their airways, but there are exceptions. About 13% (7/56) of smokers had no PMNs among the cell mixture and another 16% (9/56) had counts similar to those of nonsmokers. As alluded to, the lavage procedure may wash out only part of the PMN population and more remain behind in the alveoli.

TABLE 1
DEMOGRAPHIC AND LUNG CELL RESULTS IN NONSMOKERS AND SMOKERS

Groups	Smoking: Age (yr) Sex	Smoking: HX Pk-Yr	Smoking: Cell Count (× 10_4)	Differential (%): AM	Differential (%): PMN	Differential (%): Lymphs	Differential (%): EO-BASO*
Nonsmokers (n = 44)	27.2 ± 1.2† 27M 17F	0	19.8 ± 2.3 (3–59)‡	87.4 ± 1.3 (77–99)	1.5 ± 0.2 (0–5)	9.3 ± 0.8 (0–23)	—
Marijuana Smokers (n = 7)	20.3 ± 0.7 6M 1F	—	63.4 ± 10.0 (27–99)	88.5 ± 2.6 (76–100)	3.7 ± 0.9 (0–10)	7.7 ± 2.2 (0–20)	—
Light Smokers (< 10 pk-yrs) (n = 18)	23.4 ± 0.7 11M 7F	6.4 ± 0.6	58.2 ± 5.8 (27–110)	93 ± 0.9 (79–99)	3.3 ± 0.9 (0–17)	4.0 ± 0.5 (0–10)	—
Moderate Smokers 10 to 20 pk-yrs (n = 14)	25.8 ± 0.9 7M 7F	15.8 ± 0.9	91.1 ± 16.3 (27–234)	95.7 ± 0.7 (85–99)	2.4 ± 0.4 (1–9)	2.5 ± 0.4 (0–6)	—
Heavy Smokers (> 20 pk-yrs) (n = 20)	38.2 ± 2.3 12M 8F	41.1 ± 5.9	102.0 ± 12.1 (15–200)	92 ± 0.5 (75–99)	3.5 ± 0.6 (1–18)	3.7 ± 0.5 (1–10)	—

* Less than 1% in all differential counts (from reference 10).
† Values are mean ± SEM.
‡ Range observed.

Identification of Chemotactic Substances in Airways of Animals

As an incidental finding, an awareness that inflammatory cells accumulated in ostensibly noninfected airways and alveoli developed as we were trying to characterize respiratory lymphocytes in bronchoalveolar lavage (BAL) fluid from monkeys (11). In the course of relavaging monkeys at daily intervals to procure additional lymphocytes for T- and B-cell identification, we noted a striking increase in the percentage of PMNs in the lavage fluid. Yet there was no obvious infection or irritation in the airways of the animals, which seemed to be healthy and without evidence of pneumonitis by chest radiography or physical examination. We decided, therefore, to see if there was any substance in the BAL fluid that might stimulate or attract inflammatory cells and could account for their influx into alveoli (12). The progressive increase of inflammatory cells and the relative potency of concentrated BAL to attract these cells *in vitro* are illustrated (figure 2) from a monkey serially lavaged during a 72-h interval. Each lavage was done through a fiberoptic bronchoscope placed in the same portion of lung. In the third lavage fluid,

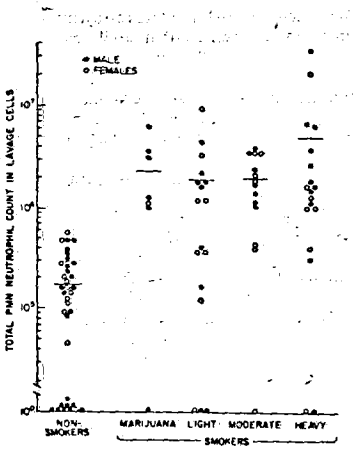

Fig. 1. Total numbers of PMNs in the respiratory cell pellets obtained by BAL are given for nonsmokers and groups of smokers. Smokers are divided according to marijuana use (no tobacco) and to history of tobacco inhalation exposure: light have < 10 pk-yr use, moderate have between 10 to 20 pk-yr use, and heavy have > 20 pk-yr use. Horizontal bars denote the mean value (from reference 10).

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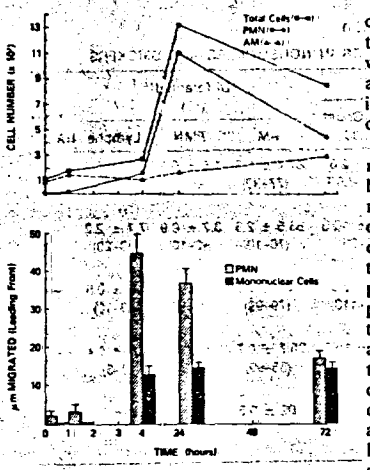

Fig. 2. A monkey underwent BAL at intervals during a 72-h period to illustrate changes in the total number of recoverable respiratory cells and in the number of PMNs and alveolar macrophages (AM) (*upper panel*). Simultaneous chemotactic activity, expressed as migration of the leading front of cells into a micropore filter, in concentrated lavage fluid was measured for 2 cell preparations (*vertical bars*) (from reference 12).

obtained 4 h after the initial one, the total respiratory count had doubled and PMNs accounted for about 45% of the cell population; when sampled at 24 h, a tenfold increase had occurred in recoverable cells of which 90% were PMNs.

When cell-free BAL fluid was assayed for chemoattractant activity that would cause directed motion of PMNs and purified monocytes into a micropore filter, considerable activity was found in the 4- and 24-h specimens. Activity in the BAL specimens was equivalent to that generated by lipopolysaccharide-activated serum and caseinate that were known to be potent chemoattractant substances. To isolate and characterize the active substance(s) in BAL, a pool of lavage fluid obtained from 5 monkeys lavaged for the fourth time after the initial lavage was chromatographed over a calibrated Sephadex G-75 column, and chemoattractant activity in the effluent fractions from the column was assessed for PMNs and mononuclear cells (figure 3). Chemotactic activity was detected in 2 areas of the elution profile: Peaks A and C, which coincided with an elution position of a 15,000-dalton marker, and Peak B, which approximated a 5,000-dalton marker. Peak B activity seemed selective for PMNs. The active fractions were pooled and characterized further. For pool A, heating (56° C for 45 min) did not diminish chemotactic activity, whereas the addition of an anti-C_3 antiserum decreased the relative activity by 45%. Such information led to the conclusion that chemotactic activity found in these chromatographic fractions of BAL was due to a complement fragment, probably C_3a. Left unaccounted for was activity in Pool B, which was unaffected by anti-C_3 or C_3 antiserum but was heat labile.

We considered that Pool B's activity might arise from a cellular source. Because lavage of normal lungs yields predominantly alveolar macrophages (>90%) and only a few lymphocytes and PMNs, *in vitro* culture of macrophages was the first choice to study. When glass-adherent macrophages were additionally activated with a phagocytic stimulus of opsonized bacteria, the culture supernatant possessed considerable chemotactic potency. Intermediate potency was obtained from adherent, but otherwise unstimulated cell cultures, but decidedly less activity was generated from alveolar macrophages that were tumbled to keep them in suspension and prevent cell adherence.

Thus macrophages seemed to produce a chemoattractant substance spontaneously when cells were allowed to adhere in cultures; however, a phagocytic stimulus quantitatively enhanced the amount. Moreover, incubation of a macrophage monolayer with cycloheximide, which putatively diminished protein synthesis by cells, largely inhibited the formation of chemotactic activity in the culture medium of bacteria-stimulated cells. Hypotonic lysis of a pellet of macrophages, however, revealed chemotactic activity in the cell lysate, indicating some storage of the factor within the cell. Chemotactic substance for alveolar macrophages was concluded to be in part preformed and stored in the cells and hence available for immediate release; however, additional factor apparently could be synthesized, providing a mechanism for sustained release.

To characterize this macrophage-derived chemotactic substance, culture media was collected after 8 h of cell stimulation, an interval that yielded the most chemotactic activity in cell supernatants, concentrated and gel-filtered through the Sephadex G-75 column as shown in figure 3. Effluent fractions with chemotactic activity, again assessed in terms of migration of the leading front of cells into a micropore filter, centered around the 400 to 420 elution volume, precisely coinciding with Peak B. Activity was selective for PMNs and ineffective for mononuclear cells or eosinophils. Heating the pooled effluent fractions diminished chemotactic activity, but anti-complement antiserum did not. This factor seemed realistically to be a product of adherent macrophages in culture and not from another cell type. The adherent cell cultures were virtually all composed of alveolar macrophages (>98%), although a few adherent lymphocytes could not be rigidly excluded; other inflammatory cells such as PMNs were absent. At the time, it was uncertain if this

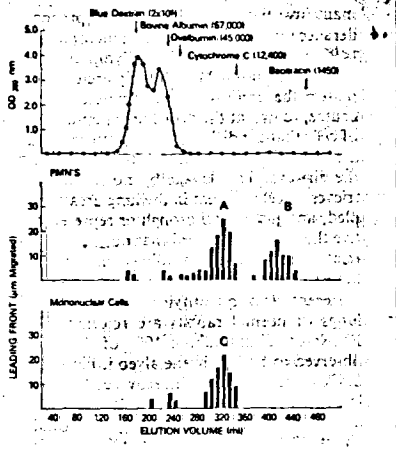

Fig. 3. The elution profile of concentrated BAL fluid from a Sephadex G-75 column is shown. Protein is located in the upper panel (OD280nm) and the elution positions of proteins with known molecular weights are given. Chemotactic activity in the column fractions, depicted on the ordinate as micrometers of migration of the leading cell front into a micropore filter, is shown for PMNs (*middle panel*) and for mononuclear cells (*lower panel*). Concentrated BAL fluid has two peaks of chemotactic activity for PMNs that eluted at 320 ml (Peak A) and at 410 ml (Peak B), and one peak of chemotactic activity for mononuclear cells that also eluted at 320 ml (Peak C) (from reference 12).

small molecular weight factor released from *in vitro* cultured alveolar macrophages was unique or possibly related to other cell-associated chemotactic factors such as dialyzable transfer factor (13) or an eosinophilotactic tetrapeptide (ECFA) (14). However, the macrophage factor consistently showed no appreciable activity for eosinophils. Moreover, the small size of the factor seemed to differentiate it from other substances with biologic activity known to be secreted by macrophage (15).

Within the same laboratory, namely the Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, Bethesda, MD, other colleagues also were looking for endogenous chemotactic substances that could produce an inflammatory response in lung tissue. Hunninghake and associates (16), working with an inbred strain 13-guinea pig system, identified a chemotactic factor generated by alveolar macrophages after phagocytosis of heat-killed *Staphylococcus aureus*. Characterization of this factor in lung lavage fluid found it to elute from a calibrated Sephadex G-75 column in the position of <5,000-dalton marker and to be resistant to heating at 56° C for 45 min, but totally destroyed after incubation at 100° C for 10 min. For *in vitro* cultured alveolar macrophages, a bacterial stimulus enhanced factor secretion, although an appreciable amount was

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produced spontaneously by the adherent cells; inhibiting protein synthesis by the cells likewise diminished the amount of chemotactic activity generated in the culture supernatant by postphagocytic macrophages. This study extended prior observations in the monkey model discussed (12) in 2 important ways. First, it demonstrated that an intratracheal injection of heat-killed *S. aureus* (10^{10} lung dose/pig) produced a significant *in vivo* PMN inflammatory response. Second, when the active factor, generated *in vitro* from macrophages, was instilled intratracheally, a significant increase in PMNs in lung BAL was found, suggesting an *in situ* role for this chemotactic substance.

Compared with the saline-injected control guinea pigs that developed about 0.5×10^6 PMNs in lung lavage, the animals that received macrophage-derived chemotactic factor had about 2×10^6 PMNs per lavage. Consistently, this factor, like the monkey macrophage factor, preferentially attracted PMNs and had no effect on eosinophils and other inflammatory cells. From photomicrographs of the lungs, obvious alveolar hemorrhage was not produced nor elicited by the chemotactic specimens. This latter finding has not been stressed enough, I believe, because the lack of appreciable erythrocyte leakage into the alveolar spaces distinguishes this mechanism of lung inflammation from that generated by the airway instillation of preformed immune complexes (17, 18) or of C_5a (19), both of which cause alveolar hemorrhage and suggest a more fulminant and possibly injurious lesion. The macrophage-induced chemotactic stimulus seemed to produce a more discreet and specific PMN accumulation in alveolar spaces.

Using the same guinea pig model, Gadek and colleagues (20) delved more thoroughly into the kinds of stimuli that induce alveolar macrophages to release the PMN specific chemotactic factor. A variety of particulates was used to stimulate adherent macrophages under tissue culture conditions that included heat-killed *S. albus*, zymosan particles, Sepharose beads, and IgG containing immune complexes. As noted in previous studies, physical adherence of macrophages to a surface was a sufficient stimulus for chemotactic factor production, but the kinetics of its formation were slow and did not reach a maximum until 18 h in culture had elapsed. In contrast, this activity after particulate stimuli developed much faster and was quite evident in the 3-h supernatant specimens.

The greatest chemotactic activity was produced by the immune complexes and IgG coated erythrocytes; intermediate activity was found with the microbial stimuli. Furthermore, if the zymosan particles were preincubated with normal serum and then fed to macrophage cultures, the output of chemotactic factor was twofold greater. The active component in serum was found to be C_3b, which complexed to the particulate antigen and probably enhanced attachment to the surface of the macrophage. Evidence suggests that C_3b was produced by the alternate complement pathway that is most likely the dominant pathway operating in the normal airway (3). These findings that IgG and C_3b containing stimuli elicit maximal output are not surprising, knowing that alveolar macrophages have special receptors for IgG Fc and C_3 (21). Likewise, specific receptor binding would ensure good particle attachment to cell membranes and facilitate phagocytosis. Chromatography on Biogel P-2 of macrophage cell culture supernatant separated chemotactic activity into 2 elution positions corresponding to molecular weights of 400 to 800 daltons.

Evident in all the monkey and guinea pig alveolar macrophage experiments was the high background of spontaneous secretion of chemotactic factor into culture medium. In part, cellular attachment to the culture chamber is responsible for activating the macrophages and increasing baseline metabolic function; preventing cell adherence by tumbling them in culture greatly diminishes the production of chemotactic activity. Although ingested particles, bacteria, and immune complexes can appreciably enhance chemotactic factor secretion, it is evident that secretion of the factor is largely nonspecific. Moreover, once liberated, this chemoattractant activity generated by macrophages may be generalized rather than local and selective. Some recent data support this view.

In our initial monkey lavage experiments (12), the same portion of the lungs was repeatedly lavaged through a main stem bronchus; other nonlavaged lobes were not systematically sampled for influx of inflammatory cells. Likewise, guinea pigs were injected intratracheally with a milliliter of fluid containing macrophage-derived chemotactic factor, rather than selective placement of the sample, and then whole lungs were lavaged to recover cells. Cohen and Batra (22), using dogs, found that repeated washing of 1 lung segment initiated an influx of inflammatory cells into unwashed segments, even in the contralateral lung. Thus, unilateral lung lavage produced a bilateral PMN response, always greater in the ipsilateral area washed but appreciable in the contralateral segment too. In fact, repeated placement of the wedged bronchoscope (5 mm size) in a sublobar bronchus produced a PMN rich influx into the area, demonstrating that actual lavage was not a necessary requirement for attracting PMNs to the alveolar surface and that a foreign body or irritant, in this instance the bronchoscope, would suffice. Minimal influx of PMNs also was noted in a couple of dogs (2/8) when the contralateral lung that had not been invaded with the bronchoscope was lavaged and cells analyzed. The experiments showed that local sublobar lung lavage had widespread alveolar effects in that repeated lavage or just placement of the bronchoscope could evoke a PMN inflammatory response in a specific lung area and in distant, removed ones as well, indicating that a general stimulus must be liberated, possibly a neurogenic or reflex one. The effect was not limited to lung parenchyma because blood leukocytosis and bone marrow release of young forms of PMNs also occurred, no doubt to supply an adequate number of PMNs for egress into lung. More than just a macrophage chemotactic factor was operant in this large-scale response. These investigators also found that monkeys responded in a similar way. Moreover, they selectively instilled concentrated lung lavage fluid into the right or left bronchus intermedius of monkeys and found that this fluid elicited a transient but 240% increase in peripheral blood PMNs, confirming a systemic effect on the bone marrow reserves from the lung focus. However, an identification of active factors present in the lavage fluid was not attempted.

Human Alveolar Macrophage Factors

As expected, the search for inflammatory mediators in the airways quickly moved to the human lung and focused on the alveolar macrophage. Lung lavage of normal volunteers produced a high yield of macrophages (> 80% of respiratory cells recovered) from cigarette smokers and nonsmokers (10). Because the monkey and guinea pig studies had identified a macrophage-derived chemotactic factor, it was reasonable to determine whether human cells made the same. Merrill and colleagues (23) found this to be the case. *In vitro* cultured alveolar macrophages produced chemoattractant activity in cell free supernatant after surface attachment. But peak activity did not occur until 22 h in culture if the cells were not stimulated further. Preventing attachment by tumbling the macrophages greatly retarded the production of chemotactic activity, but after 22 h comparable amounts of activity were present. However, when adherent macrophages were stimulated with an IgG-containing immune complex that attached to their cell surface, chemotactic activity was detected in the first supernatant sample at 3 h and in maximal amount. The quick burst of activity tapered off and was decidedly less potent in the 22 h sample. Zymosan-particles that gave a microbial phagocytic stimulus produced intermediate amounts of chemotactic activity, reaching maximal activity by 6 h, but in contrast sustained the degree of activity into the 22-h supernatant specimen. Thus the kinetics of chemotactic activity produced by the macrophage-predominant cell cultures (>95%) showed that cell adherence alone was a sufficient stimulus to cause activity and preventing adherence retarded it significantly, but did not prevent it entirely.

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Of course, mechanical agitation of the cells to prevent surface attachment should be considered a form of stimulation and is not a perfect control situation, especially when compared with the macrophages' in situ position on the alveolar surface. Lung movement obviously occurs with ventilation, but the rhythmic, less violent expansion-contraction of the alveolar surface occurring with tidal breathing and modulated by surfactant and other secretions may be far different from a rotating, plastic tube and sloshing medium. Of interest, approximately 2 wk in longterm culture was required before alveolar macrophages seemed to calm down and produce no detectable chemotactic activity in their culture medium. Poor nutrition and marginally viable cells were not the entire explanation, because cellular stimulation with IgG complexes produced maximal secretion of chemotactic activity from the 2-wk-old macrophage cultures. Like macrophages from other species, the chemoattractant substance(s) was relatively specific for inducing PMN motility and decidedly less potent for mononuclear cells.

To characterize the chemotactic activity produced by monolayers of alveolar macrophages from noncigarette smokers, supernatant specimens from unstimulated cell cultures were collected, concentrated, and chromatographed on a calibrated Sephadex column (figure 4). Eluent fractions containing chemotactic activity for PMNs centered about the elution position of a 9,500-dalton material and an approximately 1,000-dalton marker. In similar studies using supernatant from zymosan-stimulated macrophages, the same 2 peaks of chemotactic activity were detected in the elution profile, but the relative potency of the activity was somewhat different, shifting to more of the smaller molecular factor. This suggested that acute cell activation might change the relative proportions of factor secretion or indicate that the smaller substance was a breakdown product of the larger.

The relationship between these 2 possible chemotactic factors was not elucidated completely. Further analysis of the larger molecular weight factor indicated it was susceptible to proteolytic degradation with trypsin that diminished its activity. Isoelectric focusing in polyacrylamide gels of an ^{125}I trace-labeled specimen actually revealed the "homogenous" factor to disperse into a multiple band profile, but with chemoattractant activity confined to a single peak with a pI of 5.0 (peak B). Various inhibitors, including anti-C_5 and -C_3 antisera, confirmed the integrity of this 9,500-dalton chemotactic factor to be non-complement in nature. But blocking prostaglandin metabolism in the cells did inhibit early release of chemotactic activity by macrophages, and it was considered probable that some activity attributed to the small molecular weight substance could represent a lipoxygenase pathway product.

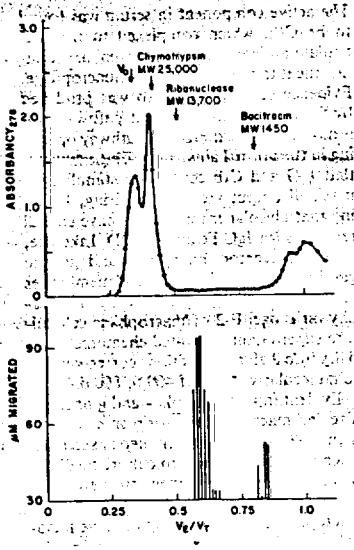

Fig. 4. Gel filtration of pooled AM culture supernates. One hundred fifty ml of unstimulated AM culture supernate from nonsmokers were concentrated to 7 ml and filtered through Sephadex G-50 SF (column dimensions 2.6 (i.d.) × 61.4 cm with gel bed volume of 324.6 ml) in phosphate-buffered saline, pH 7.2. The position of eluent fractions (4.5-ml fractions were collected) is expressed as V_E/V_T. In Panel A, the relative protein content (A_{278}) of the fractions and the elution position of calibration markers are given. In B, migration of PMNs in micrometers toward these fractions is shown. Control cell migration in the buffer was 30 μm. The column apparatus was sterile and eluent fractions were free of detectable endotoxin material (from reference 23).

Concomitantly, Hunninghake and colleagues (24) were analyzing their human alveolar macrophage-derived chemotactic factor. The results were complementary but slightly different, although the basic methodology used was generally similar. BAL from healthy nonsmokers recovered respiratory cells, principally alveolar macrophages (mean 92%), that were cultured and stimulated with a variety of particles, opsonized particulates, and immune complexes (bovine serum albumin–IgG anti-BSA antibody). Active cell supernatants were chromatographed through Sephadex G-25 or Biogel P-2 gel media in phosphate-buffered saline. Cell supernatants were generally harvested 3 h after specific stimulation for assay. Just as we noted (23), special macrophage stimulation could enhance the release or secretion of chemotactic activity in several hours; however, with extended culture (18 h), nonstimulated control cells eventually produced maximal activity. All particles stimulated chemotactic activity versus controls, but opsonization of the particles provided an additional increment, especially with heat-killed *Staphylococcus aureus*. IgG-containing complexes were particularly potent, but complement receptor attachment to the macrophages' surface was not an efficacious stimulant.

After fractionation of active cell culture supernatants, chemotactic activity was consistently found in the 400 to 600 dalton elution range of the calibrated columns. Detailed characterization of this material found it to: be stable on heating at 56° C and 100° C, exhibit stability over an enormous pH range (pH 1.0 to 12.0), resist a variety of proteolytic enzymes, contain 2 major isoelectric points (PI 7.6 and 5.2), and be extracted by organic solvents, suggesting it contained some lipid components. In terms of functional activity, the material was preferentially active for directing migration of PMNs compared with that of monocytes and eosinophils. Moreover, macrophage supernatants containing chemotactic activity induced normal human PMNs to release lysozyme and lactoferrin.

In summary, several small molecular size chemotactic factors have been conclusively demonstrated and characterized. These factors are compared (table 2) for the various species of alveolar macrophage studied. It seems that human alveolar macrophages can secrete at least 2 well-defined chemotactic factors that show selective activity for PMNs. The small < 1,000-dalton factor is in part a lipid-containing substance and may represent a lipoxygenase pathway metabolite of arachidonic acid. This possibility is relevant because one such substance, 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid, has been found to stimulate random and directed migration of PMNs and eosinophils (25, 26).

To give credence to the concept that an alveolar macrophage-derived chemotactic factor might participate in the inflammatory reaction in human lung diseases, Hunninghake and colleagues (27) sought evidence of chemotaxin secretion by alveolar cells lavaged from patients with idiopathic pulmonary fibrosis (IPF). This was a relevant disease to study because a low-grade alveolar inflammation is often present, especially in an early, cellular stage of disease. In the initial studies analyzing bronchoalveolar lavage fluid from IPF patients (28, 29), it was noted that among the respiratory cells retrieved from untreated patients, PMNs accounted for a consistent percentage (mean about 30%, 5 to 80 range observed) of the cells. A small percentage of eosinophils (about 4%) also was characteristic. Moreover, PMNs correlated with cellular reactivity in biopsy specimens (28) and with gallium lung scanning that generally reflects isotope uptake by inflammatory cells and activated macrophages (30).

A group of 15 patients with IPF, considered to be in midstage of their disease, were investigated for possible secretion of

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TABLE 2
COMPARISON OF ALVEOLAR MACROPHAGE DERIVED FACTORS

Origin	Size	Stability	Characteristics
Monkey[12]	5,000 daltons	Heat labile	Attracted PMNs not monocytes or eosinophils
Guinea pig[16]	1,500 daltons (600 d)	Heat stable	Generated after *in vivo* phagocytosis, had *in vivo* chemotactic activity
Human[24,25,27]	8,500 daltons	Sensitive to trypsin and heat stable	PMNs attracted PI 5.0
	< 1,000 daltons (600 d)	Resistant to trypsin and proteases, heat and pH stable	PMNs attracted in preference to monocytes and eosinophils; promoted release of elastase and lysozyme from PMNs
		Sensitive to serum chemotactic factor inhibitor	PI 7.6 and 5.2 in part lipid, but not HETE

alveolar macrophage-derived chemotactic factor and compared with 8 nonsmoker normal volunteers (27). After BAL to retrieve respiratory cells from both groups, the cells were submitted to Hypaque-Ficoll density centrifugation to remove PMNs; the purified mononuclear cells, principally alveolar macrophages, were cultured for 3 h after which supernatant fluids were harvested and evaluated for chemotactic activity. Various stimuli were given to the macrophage cultures in the form of concentrated BAL fluid from IPF patients, Sepharose 4B beads, and IgG coated ox erythrocytes. Results of respiratory differential cell counts in the patients showed that 9 of 15 had greater than 10% PMNs among the cells (approximately 35%), and that the other patients had less than 10%; the normal nonsmokers did not have PMNs. Once in culture, macrophages from IPF patients with > 10% PMNs in their original mixture of lavage cells spontaneously released chemotactic activity into the culture medium, whereas cells from those with fewer than 10% PMNs generally did not release activity, nor did the cells from nonsmokers.

This just may have reflected nonspecific activity by the macrophages, and an interesting control would have been secretion by other intrinsically active macrophages as obtained from sarcoidosis patients or age-matched smokers. Nonetheless, the IPF macrophages clearly generated large amounts of chemotactic activity quickly (by 3 h); when this activity was localized in the chromatogram (Sephadex G-25 gel) of the supernatant fluid, it was found in the 400 to 600 molecular weight range fractions eluted from the calibrated column. Further analysis of this material showed preferential chemoattractant activity for PMNs compared with blood monocytes, and this activity was largely extractable with organic solvents, indicating a significant lipid component to be present. Thus, Hunninghake and colleagues succeeded in identifying the same kind of small molecular weight, partially lipid-containing chemotactic factor they had previously found from normal alveolar macrophages (24).

These investigators looked further for a mechanism that could account for the secretion of chemotactic factor by IPF macrophages, an important cell component of the inflammatory, cellular milieu of active alveolitis. The stimulus seemed to be in the form of an immune complex based on the following evidence: (*1*) patients with serum titers of immune complexes generally had alveolar macrophages that released large amounts of chemotactic factor, (*2*) lavage fluid obtained from patients with immune complex titers in serum could stimulate normal macrophages to secrete the factor, (*3*) actively secreting macrophages had partial blockage of their surface IgG Fc receptors, suggesting concomitant binding of immune complexes, and (*4*) intracytoplasmic granular deposits of IgG, identified with immunofluorescent antiserum, were in the actively secreting macrophages. In BAL fluid for 9 of 10 IPF patients with actively secreting macrophages, C_{1q} binding was increased, compared with fluid from normal subjects and from IPF patients whose macrophages did not release chemotactic factor, indicating that IgG complexes were present. Enumeration of immunoglobulin secreting cells (B-lymphocytes) in BAL was not determined for these patients (31, 32), so the origin of the complexes, whether from peripheral blood or local secretion in lungs, was not known.

This study indicates that a chemotactic factor was released from alveolar macrophages of patients with IPF who were in an active phase of alveolitis with PMNs present in bronchoalveolar lavage fluid. IgG immune complexes appear to be an important macrophage stimulus. Thus an endogenous, airside chemotaxin can be released quickly within a few hours and this capability correlates with an elevated PMN count (> 10% respiratory cells). It is important that excessive chemotactic factor secretion has been found in a special disease in which some inflammatory cell reaction is a usual pathologic finding. The mechanism offered seems reasonable that chemotactic factor released from alveolar macrophages can attract PMNs to the lung. In contrast, a diminished, insufficient inflammatory response in the lungs is often noted in patients or animals that are immunosuppressed and have a propensity to develop bacterial respiratory infection. Often profound granulocytopenia and a depleted bone marrow supply of PMNs are the ready explanations. Impaired alveolar macrophages, specifically a diminished capacity to generate chemotactic factor activity, also could contribute to a depressed lung inflammatory response.

Pennington and Harris (33) examined that question with a guinea pig model in which cyclophosphamide (15 mg/kg weight/day) or cortisone acetate (100 mg/kg weight, subcutaneously) treatment was given for 7 days. Alveolar macrophages were then obtained by lung lavage and placed in tissue culture. Adherent but otherwise unstimulated cells were left in culture for 24 h and at this time (for no activity was found in 4-h cultures) cell-free supernatant was assayed for chemotactic activity for PMNs. Both treatment regimens given for a week caused approximately a 25% decrease in the potency of the macrophage supernatant to attract PMNs. It was concluded that chemotactic activity produced by macrophages could be adversely affected and this might blunt the inflammatory response. Because therapy with corticosteroids does favorably influence PMN counts and inflammation in the airways of patients with cellular forms of IPF (29, 32), suppression of endogenously produced alveolar macrophage chemotactic factor might result. For the IPF patients in the series of Hunninghake and coworkers (27), 3 patients in the high and the low intensity alveolitis groups were receiving corticosteroids when studied, but the macrophage output of chemotactic substance was not compared in these respective specimens. Thus the Pennington and Harris (33) observation has not been extended to a human situation in which corticosteroid therapy was given.

Other Chemotaxins, Potentially of Alveolar Macrophage Origin

A large array of biologic substances has been attributed to synthesis and secretion by macrophages (15, 34, 35). In a recent count, over 50 things had been identified (34) and it is probable that new entries will be added. However, only 2 substances in the current list seem to have clear chemotactic activity that would qualify them for serious consideration as other endogenous chemotaxins produced by alveolar macrophages. They are C_5a and certain leukotrienes derived from arachidonic acid metabolism.

The role of the complement system in host defense of the airways and alveolar surface remains controversial. Properdin

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Factor B (36) and a number of other complement components have been identified in lung lavage fluids (9, 29, 37) which support the concept that alternate pathway activation may be important in the lung. We reviewed this topic in detail in Reference 3. Interest focuses primarily on C_5a and C_5a des arg, considered to be the most potent inflammatory mediators and chemotactic molecules liberated by the complement cascades. These are capable by themselves of causing acute airway hemorrhage and exudation after intratracheal instillation (18, 19, 39, 40). If direct activation of alternative complement pathway actually occurs in the airways, then it is reasonable to postulate that a variety of inhaled or aspirated particles and microbes that gain access to the respiratory tree can trigger the complement cascade; C_3 and C_5 plus other intermediate components like the trimolecular complex C_{567} could be liberated and produce a number of fragments with chemoattractant properties. Quantitatively, C_5 derivatives seem to be the most effective and potent of the complement group.

Instead of generating one of these phlogistic fragments from the complement cascades, the precursor component normally may be present in airway lining secretions. We certainly found C_5 activity in concentrated BAL fluid obtained with serial lavage of ostensibly normal monkeys (12). Kolb and coworkers (41) also found antigenic and functional C_5 activity in concentrated BAL fluid from cigarette smoke-exposed and normal nonhuman primates (baboons). C_5 hemolytic activity in BAL fluids actually was almost twofold higher than in corresponding serum samples (C5H50 titers per albumin concentration were about 800 in BAL in contrast to mean values of 450 in serum), and resistant to inactivation by exogenous trypsin. These results indicated that the intact C_5 precursor molecule is present in the alveolar compartment. In contrast, preliminary studies looking for C_5 in human lavage fluids from normal nonsmokers (42) have noted a paucity of this factor, compared with serum, raising the probability that appreciable diffusion of C_5 from the vascular space into the lower respiratory tract must occur before a sufficient amount is present for activation.

Another possibility is that complement components are directly added to respiratory secretions from local synthesis in the lung; some evidence supports a cellular source for this. Scherzer and colleagues (43) have found that *in vitro* cultured human alveolar macrophages synthesize and secrete into cell supernatant fluids, functional C_3 and C_5, as assessed by a hemolytic assay and further identified antigenically. Pennington and coworkers (44) also have assessed the production of complement factors by *in vitro* cultured human alveolar macrophages; specifically, they looked for C_2, C_3, C_4, C_5, and factor B. These macrophages were lavaged from patients with a variety of diseases including sarcoidosis, squamous cell carcinoma, and recurrent pulmonary infections. Most cell specimens produced functional C_2 and factor B, and several made C_3; none produced C_4 and C_5. It seems evident that cultured alveolar macrophages can spontaneously produce a variety of functionally active complement components that are linked with the classical and with the alternate pathways. It is plausible to believe that the cells can add these components directly to the fluid milieu of the alveolar surface and hence be available to interact with deposited, inhaled debris and micro-organisms. Questions still left unanswered are the quantitative nature of the secretion, whether it is regulated or spontaneous, and how specific the response can be when stimulated by phagocytosis or immune complexes attaching to the cell's surface. Moreover, C_5 does not seem to be the sole complement component produced and it may not be made in predictable amounts.

Considerable interest is focusing on other macrophage products that have potential phlogistic activity: platelet-activating factor and certain products of lipoxygenase pathway of arachidonic acid metabolism. Because this work is just emerging like the complement work, an exhaustive review of largely preliminary publications is premature. The leukotriene products, derived from oxidative metabolism of arachidonic acid along the lipoxygenase pathway, have spasmotic properties that can cause bronchospasm, and one of the principal mediators of allergic Type I asthma, a slow reacting substance of anaphylaxis (SRSa), has been found to contain 3 leukotrienes: LTC_4, LTD_4, and LTE_4 (45, 46). Initial evidence suggests that alveolar macrophages from rats (47, 48) may secrete small amounts of SRSa and thereby be an alternate source of this mediator. On the basis of work with human PMNs, stimulation will produce several lipoxygenase products that have chemotactic properties for other inflammatory cells, namely 5-monohydroxyeicosatetraenoic acid (5-HETE), and 5,12-dihydroxyeicosatetraenoic acid (LTB_4). Such products have not been identified conclusively in alveolar macrophages; however, the low molecular weight chemotactic factor released from human alveolar macrophages, as characterized by Hunninghake and colleagues (24, 27), is partially lipid in nature and may constitute part of a HETE-type moiety. LTB_4 is, perhaps, of even more interest for it is a potent chemotactic factor with activity that is comparable with C_5 (49). Undoubtedly the potential role of these substances (50) must await their identification in appropriate cells that can initiate the inflammatory reaction in the airways.

Perspective: Role of Endogenous Chemotaxins

As already mentioned, the capability of generating an inflammatory reaction in the alveoli and on distal airway surfaces of the lung is a fundamental element of normal respiratory host defense. Yet full mobilization of the reaction is seldom needed; overt pneumonia, as an example, is an infrequent occurrence in the healthy person. Thus individual, well-controlled components of the overall defense system suffice in most instances to clear or contain foci of debris and infection.

Exclusion of foreign substances inhaled with inspired air is obviously very efficient, and the usual surveillance mechanisms operant in the nose, throat, and conducting airways are effective in intercepting these things. On the air exchange surface, alveolar macrophages are assumed to be the scavenger and phagocytic cell front line that cleanses this area, but this may not always be the case as the particular particle or microbe can be handled in different ways. Amply shown in aerosol-exposed animal models that feature reproducible deposition of bacteria in the lungs (51–53), certain bacteria, such as *Streptococcus sanguis* and *Staphylococcus aureus*, are contained by alveolar macrophages; whereas others require a combined macrophage and PMN response, as found for *Streptococcus salivaris*, or elicit a predominantly PMN response in the case of *Neisseria catarralis* or *Klebsiella pneumoniae* and *Escherichia coli*. In general terms (54), the gram negative organisms containing lipopolysaccharide cell wall components evoke more inflammatory response with PMNs than gram-positive ones, a distinction of some relevance in assessing which factors are important in initiating the host's inflammatory reaction (figure 5).

Once in the alveolus, a bacterium might stick to the lipid (surfactant) proteinacious lining secretions that are thinly spread over the alveolar surface and may soon be ingested by a macrophage. However, several possible things could happen. The bacterium, an encapsulated one of a gram-positive (GR+) or gram-negative (GR−) variety, could become coated with a mixture of immunoglobulin (? antibody), surfactant, and fibronectin, or interact directly with available complement pathway proteins. Proteases (and lipopolysaccharide from the gram-negative organism) from the bacterium could be liberated as a consequence and activate or degrade any parts of the mixture just mentioned. What ensues might not always be predictable. The complement sequence could be activated (alternate pathway) and C5a or C_{5a} desarg generated directly from a precursor component, or opsonic antibody and fibronectin could facilitate phagocytosis by the macrophages that would lead to bactericidal killing of the microbe. Once stimulated, the macrophage could enhance its secretion of chemotactic factors, including HETE and possible leukotrienes, or produce complement components. C_{5a} once present can also stimulate macrophages to release chemotactic factor and lysozomal enzymes (39). The net effect

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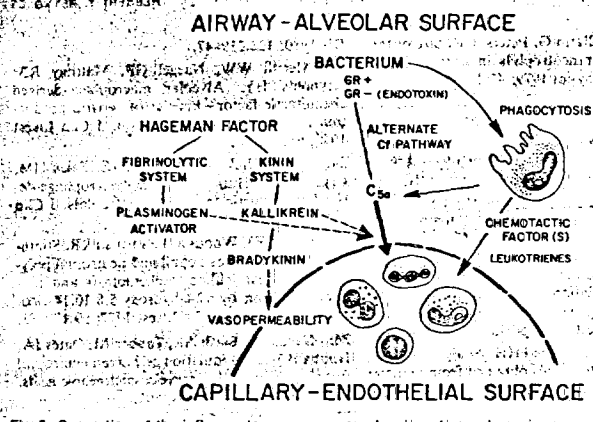

Fig. 5. Generation of the inflammatory response can be viewed as a dynamic process involving both sides of the air-blood interface, but in this illustration the stimulus originates on the airway-alveolar surface and spreads to include cellular and humoral elements in the adjacent capillary and on the endothelial surface. The airway stimulus could be multiple things, but an inhaled or aspired bacterium is an appropriate one (see text for details).

of all these things could be an appreciable increase locally in the alveolus of several factors that possess potent chemo-attractant activity. Either maintenance of a large concentration gradient across the air-blood interface or diffusion of these factors into the vascular compartment could begin the influx of other inflammatory cells into the alveolus. Because the macrophage chemotactic factors studied are rather selective for PMNs, PMN phagocytes seem to be preferentially attracted from the circulating-marginated blood pool. Thus a combination of local macrophage and exogenous PMN phagocytic firepower can be mobilized to the alveolus.

Obviously, many other components of the inflammatory response are present in the alveolus, and mechanisms that could be responsible for them are still poorly understood. Evidence suggests that proteases liberated from PMNs or mast cells can activate a number of Hageman factor-dependent pathways that can initiate the complement sequence, kinin system, and fibrinolysis in addition to the intrinsic pathway of coagulation usually associated with this factor. Whether all of these pathways are operant in the lung is not known conclusively. However, a number of intermediate products formed from these pathways could be relevant in the inflammatory reaction. Plasminogen activator and kallikrein have chemoattractant activity; others have vascular effects that could be important. Products of the kinin system such as bradykinin can alter vascularpermeability that could cause leakage of fluid from the plasma compartment into alveoli. "Inflammation" tends to emphasize cellular components of the reaction, but fluid elements are perhaps of equal importance. Once selective permeability of the endothelial-epithelial surfaces of the blood-air barrier is breached, possibly governed by vasoactive mediators, wholesale transudation of plasma proteins occurs, and high concentrations of many substances important in the inflammatory process, such as complement components, can accumulate.

What becomes crucial is to determine where the fine-tuning of the reaction occurs. What recognizes the specie of bacteria, for example; what causes the switch in phagocytic cell response between macrophages and PMNs; what amplifies the reaction and mobilizes blood cell and plasma resources, and what calls a halt to things once the invasion is contained and gets clean-up and repair under way? In other words, is the reaction a selective and qualitative one with intrinsic control? Alternatively, there may be nothing selective about the inflammatory sequence in the lung, and it is just a quantitative response.

After a certain degree of local injury, the response escalates, albeit in a rather predictable way. Obviously, it is an oversimplification to consider that just one component of lung or systemic host defense is in charge of everything, because biology is more clever than this and several levels of regulation are invariably available. However, the tissue macrophages in the lung are a plausible cellular entity to focus on as potentially a very important point of control in the inflammatory reaction just outlined. What makes this consideration at all feasible stems from the extraordinary versatility of the macrophage itself, which can be at once an effector cell and phagocyte par excellence, and an effector cell capable of secreting a bewildering array of biologic substances (15, 34, 35). Chemotactic factors, complement components, and leukotrienes, selectively considered in this report, constitute only a small number of substances these cells can create.

A small percentage of PMNs is usually found among the respiratory cells retrieved by BAL from lungs of nonsmokers (about 1%) and cigarette smokers (about 3%); since lavage may not completely dislodge all the PMNs, the actual *in situ* number on the alveolar and peripheral airway surfaces is probably greater. But why PMNs should leave their circulating blood pool and migrate into the lung parenchyma and onto the air exchange surface, in the absence of an overt stimulus to attract them, is uncertain. In smokers who inhale burning cigarette gases and particles, the added airway debris probably accounts for the influx of PMNs and the huge increase in the number of macrophages; whereas, in normal nonsmokers small foci of bacteria and occasional particles landing on the alveolar surface could serve as a continuing, low grade stimulus for a very modest inflammatory cell influx. On the other hand, alveolar macrophages are clearly capable of secreting chemotactic factors that seem to preferentially attract PMNs and they could provide one such alveolar-side stimulus. *In vitro*, however, the secretion of chemotactic factors seems nonspecific and can be generated simply by cell adherence and prolonged culture, although certain phagocytic and immune complex stimuli can greatly enhance output.

In vivo, things might be quite different. Macrophages are dispersed throughout the alveoli surface and one cell patrols several alveoli; other macrophages are present in the interstitial and structural areas of the alveoli, presumably undergoing maturation before emerging on the air exchange surface. It is not known if the young, maturing macrophages are as capable as the alveolar ones of secreting chemotactic factors. Whereas, *in vitro*, several million macrophages, activated by having to attach to a foreign surface, seem to produce spontaneously a lot of chemotactically active material in cell culture supernatant, the actual concentration of factors liberated by a single macrophage into its immediate surroundings could be quite minute. Such an amount might not be sufficient to actually attract PMNs into the alveolar space, but the factor could seep into the capillary endothelium and promote sticking of PMNs to that surface. Hence a low level of tonic secretion of chemotactic factors by airside alveolar macrophages might be a mechanism for localizing inflammatory cells such as PMNs in adjacent lung capillaries; more potent stimuli may be required to draw them into lung parenchyma. Within several hours, macrophages can maximally secrete chemotactic factors in response to a phagocytic challenge or IgG containing immune complexes, a situation that might need to occur before PMN influx takes place. In addition, other stimuli including complement fragments, arising independently, may need to act together to promote inflammation. Conceivably, macrophage chemotactic fac-

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tors have a dual role: (*1*) to diffuse out of the alveoli surface and aid with margination of PMNs in the vasculature, and (*2*) to accumulate in sufficient concentration intra-alveolarly to create a gradient that will attract cells across the blood-air interface onto the alveolar surface.

The importance of macrophage-derived factors in promoting chronic lung diseases that have persistent low-grade PMN inflammation is likewise unclear. Chronic bronchitis and emphysema associated with cigarette smoking feature elastolytic tissue damage that occurs in part from a combination of excessive leukocytic elastase released from PMNs and inadequate neutralization of it by the protease inhibitor, alpha$_1$ antitrypsin, that is rendered nonfunctional by the smoke (55, 56). Not only do such subjects have a huge increase in the number of alveolar macrophages, but their macrophages are generally more active biochemically (57), and tonic secretion of chemotactic factor seems to be greater. PMN influx into lung tissue might be aggravated. This seems to be a mechanism in patients with idiopathic pulmonary fibrosis who have a high percentage of PMNs in their alveoli and airways, but in this disease an additional stimulus such as an immune complex might be required to promote chemotactic factor secretion.

Nonetheless it seems reasonable to consider ways of blocking the biologic activity of macrophage chemotactic factors. A means of selectively suppressing the macrophage does not appear obvious, although corticosteroids and other immunosuppression may generally reduce chemotactic factor production (33). Alternatively, development of an inhibitor polypeptide or a specific monoclonal antibody might be feasible ways to block this chemotactic activity exogenously. Hopefully, some form of chemotactic inactivator will emerge in the future as a possible therapeutic agent to modulate inflammation.

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The Presence of Neutrophil Elastase and Evidence of Oxidation Activity in Bronchoalveolar Lavage Fluid of Patients with Adult Respiratory Distress Syndrome[1,2]

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SUMMARY Evidence obtained by biochemical analysis of BAL fluids from patients with ARDS indicates that at least two important pathogenic events take place in the pulmonary tissues. These are the release of elastase from neutrophils and the generation of oxidants. Both events can lead to severe pulmonary injury, as has been demonstrated in laboratory animals and isolated perfused lungs.

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We examined bronchoalveolar lavage (BAL) fluids from patients with adult respiratory distress syndrome (ARDS) for the presence of mediators of the inflammatory injury. The method of obtaining BAL fluids, together with a critique of the procedure, has been published (1).

In the initial experiments, aliquots of BAL fluid were incubated with (^{125}I) components of the contact (Hageman factor, prekallikrein, plasminogen, and high molecular weight kininogen) and complement system of proteins to determine whether activators of these systems could be found (figure 1). BAL fluids of ARDS patients contained protease(s) that cleaved 4 components of the contact system, and C3, C4, C5, and factor 8 of the complement system, (data not shown). Plasma of the patients, diluted to the same concentration of protein as the BAL fluid, was not active.

The major protease of the BAL fluids was identified by use of inhibitors, to determine an inhibitory profile, and by specific methods (1). The proteolytic activity was inhibited entirely in 10^{-4} M diisopropylphosphofluoridate (DFP) but not by chelation with 1,10 phenanthroline. Trasylol was also inhibitory, but only in high concentration. CI inhibitor was inactive, but while normal plasma could inactivate the enzymes(s), α-1-PI (A1AT)-deficient plasma did not. Addition of purified α-1-PI to the deficient plasma reconstituted the inhibitor capacity. This suggested that leukocytic proteases could be involved.

The protease was then found to lyse insoluble elastin and cleave methoxysuccinyl (ala)$_2$ pro val pNA, both substrates for

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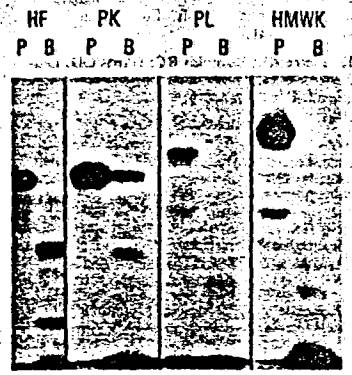

Fig. 1. SDS-PAGE with β-mercaptoethanol of ^{125}I-labeled Hageman Factor (HF), prekallikrein (PK), plasminogen (PL), and high molecular weight kininogen (HMWK) after incubation (20 min 37° C) with 20 μl of plasma (diluted 1:70 with sterile saline) (P) or 20 μl of bronchoalveolar lavage fluid (B) of patient 7. Cleavage of each protein occurred after incubation with lavage fluid as described in the text. A radiolabeled contaminant is present in the HF preparation in P immediately below the major band of HF, and a small amount of spontaneously cleaved plasminogen and HMWK was observed in the preparations that were untreated or exposed to plasma (P). (From McGuire, *et al.*, J Clin Invest 1982; 69:543–53.)

neutrophil elastase. Because chelation with 1,10 phenanthroline failed to block proteolytic activity and, as noted, DFP was inhibitory, the elastase was differentiated from macrophage enzyme. Monospecific antibodies to neutrophil elastase reacted with antigen in the BAL fluid of ARDS patients (figure 2) and inhibited the protease activity. The enzyme was therefore identified as neutrophil elastase (NE).

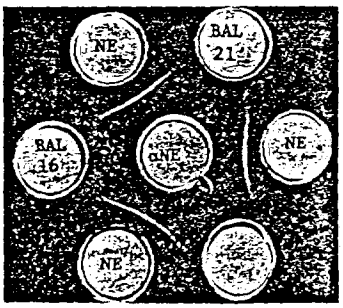

Fig. 2. Double diffusion immunoprecipitin analysis of the presence of neutrophil elastase (NE) antigen in two cases of BAL from patients with ARDS. Coalescence is noted between these precipitin bands and those developing with purified NE. The center well contains anti-NE (αNE). The small line of precipitation near the center well and between wells containing BAL 21 and BAL 16 fluids probably represents a complex of elastase and α_1-PI inhibitor. (From McGuire, *et al.*, J Clin Invest 1982; 69:543–53.)

In order to determine if the NE was the predominant enzyme, [^{3}H] DFP was added to BAL fluids and the incubated mixture subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE), followed by autoradiography. The autoradiographs revealed [^{3}H]DFP binding to proteins exclusively at 28,000–31,000 Mr, i.e., in the range of NE. These combined data suggested that NE was the dominant protease in the BAL fluids.

NE activity was found in BAL fluids of 17 out of 24 patients with ARDS. Inactive NE was detected in an additional 4 patients immunologically. Only single samples of BAL fluid were available from the patients not containing NE activity. Because during the course of ARDS BAL fluids at times contained active NE, but at other times did not, it is possible that lavage fluids of those patients not demonstrating NE would have contained NE at another time in the course of the illness.

The finding of NE in BAL fluids was not unique to this disease. While BAL fluid of 7 normal individuals did not contain NE, 2 of 27 patients with presumed noninflammatory lung disease and 17 of 37 patients with bronchial pneumonia were found to contain NE. Thus the presence of NE in the lung fluids is not diagnostic of ARDS. Indeed, the data suggest that inflammation of ARDS patients cannot, at present, be distinguished biochemically from other pulmonary inflammatory diseases.

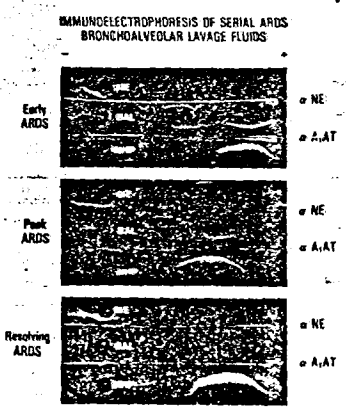

Fig. 3. Immunoelectrophoresis of BAL fluids from a patient with ARDS at three times during the course of illness. NE = purified neutrophil elastase; NHP = normal human plasma; αNE = anti NE; A1AT = anti-α_1-PI. The precipitin arc developing between anti-NE and NE in the BAL fluid in the early and peak times in ARDS was seen displaced to the beta region of the plate, opposite a slowly migrating portion of the α_1-PI. At the peak of disease, a small precipitin band of free NE was detected (seen between the top of the well and the letters BAL).

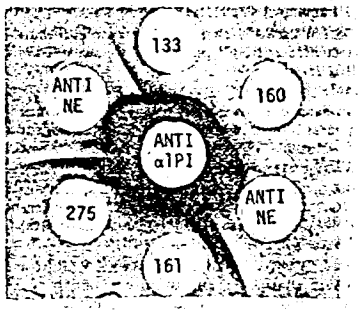

Fig. 4. Two-dimensional immunodiffusion analysis of neutrophil elastase (NE) and α_1-PI in BAL fluid of patients with ARDS (Patient nos. 133, 160, 161, 275). As noted especially in the precipitin band between wells containing anti-NE and BAL fluid from patients 275 and 161, the NE-anti NE precipitin band coalesces with the precipitin band that developed between the BAL fluids and anti α_1-PI. The coalescence indicates complexing of α_1-Pt and NE.

Similar data have been obtained by Lee *et al.* (2). These authors observed the presence of elastase in 12 of 23 patients with ARDS. An inverse correlation between active NE and α-1-PI was noted by these investigators.

Analyses were conducted to determine in what state the NE existed as an inactive protein. Immunoelectrophoretic analysis revealed that NE in these BAL fluids migrated in the β region rather than in the γ region as did the native molecule. The position of the NE was identical to that of a portion of the α-1-PI, which migrated more slowly toward the anode in the electrophoretic field (figure 3). Two-dimensional immunodiffusion analysis revealed that the NE and α-1-PI were complexed (figure 4).

Of great interest was the observation that the unbound α-1-PI in the BAL fluids was also inactive. Assessment of activity of the α-1-PI in the BAL fluids of ARDS patients revealed considerable or complete loss of activity (figure 5). Further analyses were therefore conducted to determine the cause of inactivation of the unbound α-1-PI. The immunoelectrophoretic position of the free, inactive α-1-PI suggested that the molecules were not greatly changed in isoelectric point. To determine if cleavage of the α-1-PI molecules had occurred that might account for inactivation, α-1-PI in BAL fluids in which the inhibitor was totally inactivated was adsorbed to anti-α-1-PI insolubilized to Sepharose 4B and, after washing, eluted in SDS and subjected to SDS-PAGE. In 5 of 6 samples so treated, the inactive α-1-PI was observed to retain its native size of 52,000 daltons. In the sixth, cleavage of the α-1-PI was apparent with a 47,000 Mr fragment being observed. Thus, while proteolytic cleavage may occur, examples of uncleaved, inactive α-1-PI were apparent.

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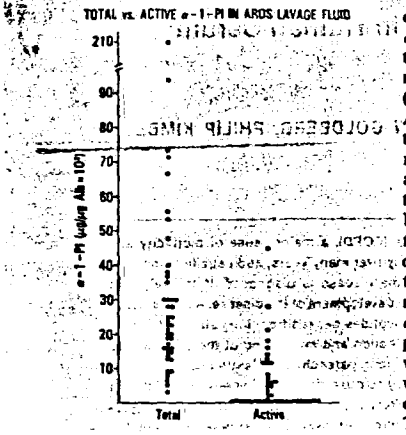

Fig. 5. Total quantity and activity of α_1-PI in BAL fluid of patients with ARDS. Total activity was obtained by quantitative immunodiffusion (Mancini) assay and activity of the α_1-PI by inactivation of porcine pancreatic elastase (*see* reference 5).

Oxidation of the α-1-PI

Another possible mechanism of inactivation involved oxidative conversion of methionyl residues in the reactive site to methionyl-sulfoxide, a reaction shown previously to inactivate the inhibitor (3, 4). We investigated this possibility employing 2 techniques. In the first, the method developed by Johnson and Travis (3, 4), potentially oxidized, inactive α-1-PI was exposed to porcine pancreatic elastase. If inactivated by oxidation, a 47,000 Mr cleavage product results and complexing of the porcine elastase by the α-1-PI does not occur. This proved to be the case in BAL fluids of patients lacking α-1-PI activity but having α-1-PI demonstrable by immunologic techniques (5).

In a second, more direct method of demonstrating oxidative inactivation of the α-1-PI (6), we exposed the BAL fluids to dithiothreitol in the presence of the enzyme methionyl sulfoxide peptide reductase (kindly supplied by Drs. Harvey Carp and Aaron Janoff). Of 9 BAL fluids treated in this manner, 8 showed partial or complete restoration of α-1-PI activity. SDS-PAGE analysis of the α-1-PI (isolated by adsorption onto antibody-coated Sepharose 4B beads and eluted in SDS) prior to exposure to reducing agent was performed in order to determine why α-1-PI activity was only partially restored in some of the cases and not restored in one. In each of these cases, α-1-PI was observed that was either complexed with NE, having a Mr of 80,000, or was cleaved to 47,000 Mr. In those cases in which complete or partial restoration was observed, 52,000 Mr α-1-PI was present (5). Thus restoration of activity of the α-1-PI resulted from exposure of the BAL fluid to reducing agent in the presence of a reductase as long as free, 52,000 dalton α-1-PI was available.

In order to determine if the α-1-PI had been inactivated systemically or in the lung of ARDS patients, the specific activity of α-1-PI in the plasma was assessed. In 14 of 22 cases, the α-1-PI was greater than 90% active (5). Thus inactivation of the α-1-PI in the lung in these cases resulted from oxidative reactions occurring in the pulmonary tissues. In the remainder, 10 to 50% inactivation occurred, suggesting that either systemic inactivation of α-1-PI had occurred or that α-1-PI was inactivated in the lung and was returned to the circulation in that form.

The experimental establishment of oxidative inactivation of α-1-PI is of critical importance, not only because the major control protein of neutrophil elastase is compromised, but also because this finding indicates the generation of oxidants *in vivo*. Because the presence of extracellular oxidants is known to injure lung tissues (7, 8, 9), its demonstration in the lungs of ARDS patients may reveal the existence of an important pathogenetic mechanism in this disease.

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Immunologic Measurement of Elastin-Derived Peptides in Human Serum[1-3]

UMBERTO KUCICH, PAUL CHRISTNER, MICHAEL LIPPMANN, ALAN FEIN, ALAN GOLDBERG, PHILIP KIMBEL, GEORGE WEINBAUM, and JOEL ROSENBLOOM

SUMMARY Chronic obstructive pulmonary disease (COPD), a major cause of morbidity and death in the smoking population, develops insidiously over many years, and usually significant impairment of lung function has occurred before the disease is diagnosed. It is likely that destruction of the elastic fiber is a prerequisite for the development of the disease, and it is possible that immunologic identification in the serum of peptides derived from lung elastin degradation might be an effective approach to the early detection and monitoring of the disease. We prepared antibodies to peptides derived from human lung parenchymal elastin and used these antibodies in an enzyme-linked immunosorbant assay to quantitate elastin-derived peptides in the serum of 39 normal control nonsmokers, 33 smokers with normal lung function, and 40 patients with COPD. On average, statistically significant higher levels of elastin-derived peptides were found in the normal smokers and COPD patients compared to the controls. Further work with larger numbers of subjects is necessary to determine whether such a test is effective in identifying those individuals who are at risk of developing COPD.

AM REV RESPIR DIS 1983; 127:S28-S30

Introduction

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and death in the smoking population. The disease usually develops insidiously over many years, and it is not until lung function has been significantly impaired that the disease can be diagnosed with certainty by radiologic and pulmonary function tests. A quantitative test that is capable of identifying the presence of lung damage at an early stage, before symptoms develop, could be useful in identifying those people who are at risk and possibly in monitoring the progression of the disease.

The connective tissue protein elastin is largely responsible for maintaining elasticity of major blood vessels and lung tissue. In experimental animal models of emphysema, the major emphasis has been on the destruction of the mature elastic fiber by selective proteases administered by aerosol or intratracheal instillation. In these systems there is a strong correlation between the production of emphysema and the cleavage of insoluble amorphous lung elastin (1-5). In humans it is also likely that destruction of the elastic fiber is a prerequisite for the development of the disease.

One possible approach to the problem of early detection and monitoring disease development is the immunologic identification of peptides derived from lung elastin degradation that may appear in the serum. As a first step in exploring the possibility, we obtained antibodies in rabbits against peptides prepared by oxalic acid or dog neutrophil elastase digestion of purified dog lung parenchymal elastin, and used these antibodies to identify and quantitate elastin-derived peptides in the serums of dogs in which emphysema had been induced by the aerosol administration of porcine pancreatic elastase (6).

In the present work we have prepared antibodies to peptides derived from human lung parenchymal elastin and used these antibodies in an enzyme linked immunosorbent assay (ELISA) to quantitate elastin-derived peptides in the serums of normal control nonsmoking individuals, smokers without evidence of disease, and individuals with COPD. The data suggest that among the three groups, the COPD patients had the highest levels whereas the normal nonsmokers had the lowest. Further investigations with larger numbers of subjects are necessary to determine whether the test will be useful in the study of human emphysema.

Methods

Purification of Elastin and Antibody Production

The amorphous component of human lung elastin was prepared by minor modification (7) of the procedure of Lansing and coworkers (8), and amino acid analysis of the preparation showed that it was highly purified elastin. Enzymatic digests of the elastin were prepared by incubation with purified human neutrophil elastase (HNE), 1 μg enzyme per mg amorphous elastin, for 24 h at 37° C in 0.05 M Tris, 0.14 M NaCl, 0.02% Na-azide pH 8.0. The reaction was terminated by the addition of 5.0 μl of 0.1 M diisopropyl fluorophosphate per ml incubation, the reaction mixture centrifuged and the supernatants used directly as antigens. The concentration of the HNE-derived elastin peptides was determined by the Lowry method (9), using bovine serum albumin as the protein standard.

New Zealand white rabbits weighing 2.5 to 4 kg were used for antibody production. Each rabbit received 0.5 mg of the solubilized elastin in a total volume of 0.5 ml of phosphate buffered saline (PBS) with an equal volume of complete Freunds adjuvant. The antigen was administered subcutaneously with multisite injections. Subsequent immunizations consisted of 0.25 mg of elastin peptides delivered weekly for at least 2 months. Seven to 10 days after the last injection, the animals were bled by venipuncture, the serums collected by centrifugation, and stored at 4° C in 0.02% NaN_3 until ready to use. Antibody titers for each animal were determined by a passive hemagglutination assay previously described (6).

Enzyme-Linked Immunosorbent Assay

Prior to the assay, the IgG fraction of both preimmune and immune serums was prepared by precipitation with 35% $(NH_4)_2SO_4$. The precipitate was collected by centrifugation at 15,000 g for 15 min, suspended in PBS, pH 7.2, and dialyzed exhaustively in the cold against the same solution. The dialyzed proteins were stored at 4° C at a concentration of 1 mg/ml in PBS, containing 0.05% NaN_3. Protein concentration of the partially purified antibody was determined by measuring the absorbance at 280 nm using an extinction coefficient, $E^{1\%}_{1cm}$, of 14. All subsequent dilutions were made from the above stock solutions.

The assay was conducted essentially as described by Engvall and Pearlmann (10) with slight modifications (11). Polystyrene tubes (11 × 55 mm) were coated with HNE-human lung elastin peptides by incubation with 2.0 μg/ml peptides at 37° C for 24 h in 0.1 carbonate pH 9.6 containing 0.02% NaN_3, rinsed with PBS, and stored at 4° C

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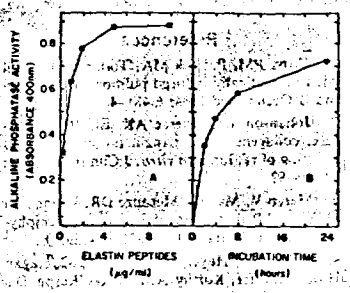

Fig. 1. (A) Binding of elastin peptides. Polystyrene tubes were coated for 24 h at 37° C with increasing concentrations of elastin peptides derived by digestion of human elastin with human neutrophil elastase, and the coated tubes incubated with 20 μg/ml specific antibody for 3 h at room temperature. The tubes were washed and ALK•PHOSPH-IgG at a 1:300 dilution was added, the tubes washed, and alkaline phosphatase activity was determined by addition of p-nitrophenyl phosphate. (B) Tubes were coated with 2 μg/ml peptide for various times at 37° C and the binding of antigen determined as in (A).

until used. Twenty micrograms of preimmune IgG and either specific antibody alone or specific antibody plus soluble antigens were preincubated at 4° C overnight in glass tubes and each solution was transferred quantitatively to precoated polystyrene tubes. The amount of specific antibody bound was quantitated by the addition of goat antirabbit IgG, to which alkaline phosphatase was covalently linked (ALK - PHOSPH-IgG) followed by reaction with its substrate, p-nitrophenyl phosphate. After 30 to 60 min incubation, the color reaction was terminated by the addition of 0.1 ml of 1.0 N NaOH and the extent of reaction measured spectrophotometrically at 400 nm. The color reaction is dependent on the amount of the bound ALK - PHOSPH-IgG which is, in turn, a measure of specific antibody uptake. A standard curve was generated using known amounts of elastin peptides: the higher the concentration of competing antigen, the greater the inhibition of color development. Subject serums were incubated with a fixed amount of specific antibody and the concentration of peptides determined from the inhibition of the reaction compared with that obtained with the standard curve. Two different amounts (25 and 150 μl) of each serum, run in triplicate, were tested.

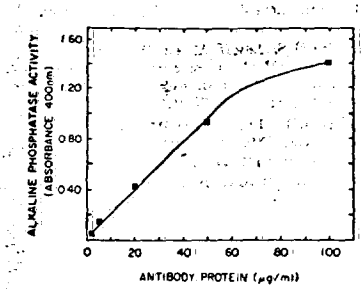

Fig. 2. Titration of elastin-specific antibody. Tubes coated with 2 μg/ml peptides were incubated for 3 h with various concentrations of elastin-specific antibody, the tubes washed, and alkaline phosphatase activity measured as in figure 1.

Patient Population Selection

A total of 109 persons were studied. They were distributed into three groups: Group I: normal nonsmokers (n = 36), Group II: normal smokers (n = 33), and Group III: COPD patients (n = 40), who were either ex-smokers or continued to smoke. Spirometry and lung volumes were determined in each by using the M-800 Autobox System produced by SRL Inc. The results were interpreted using the Intermountain Thoracic Society criteria (12). In addition, each patient filled out a detailed smoking history questionnaire, and a complete history and physical was performed on each.

Results

Development of Assay

To study the characteristics of the binding of the elastin peptides, polystyrene tubes were incubated with various concentrations of the peptides, and the extent of binding was measured using saturating amounts of specific antibody and goat antirabbit IgG. Binding was linear up to about 2 μg/ml with comparatively little additional binding at higher concentrations (figure 1A). This result suggests that maximal tight binding was reached at approximately 2 μg/ml. Binding was essentially complete by 24 h (figure 1B). Therefore, for all subsequent experiments, tubes were coated by incubation with 2 μg/ml peptides for 24 h at 37° C.

To determine the approximate concentration of specific antibody to be used in the competitive ELISA, a range of antibody concentrations was tested. A linear response was obtained up to about 60 μg/ml (figure 2). Therefore a limiting concentration of 20 μg/ml was selected for further use. A typical standard curve, illustrated in figure 3, demonstrates that the assay is useful down to about 5 ng of peptide.

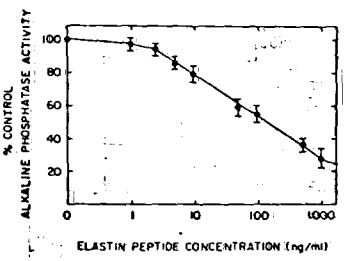

Fig. 3. Standard curve of the enzyme-linked immunosorbent assay. The competition assay was carried out as detailed in *Methods* in order to generate the standard curve. Triplicate determinations were carried out at each concentration and the bars indicate the standard deviation.

Analysis of Serum Samples

A summary of the characteristics of the three groups of subjects studied, from whom serum sample were collected, is given in table 1. As expected, the COPD patients were older, smoked longer and in greater quantity than the other 2 groups. The mean $FEV_1/FVC\%$ of 50 in Group III is consistent with moderate obstructive lung disease, whereas the $FEV_1/FVC\%$ is normal in the other two groups (> 75% is normal). The content of reactive peptides in the serum samples was quantitated using the ELISA.

The samples were assayed in a blind fashion and each was tested at a minimum of 2 dilutions, with each dilution tested in duplicate. Samples with less than 5 ng/ml were scored as zero, and only those samples that were self consistent were retained in the study.

The concentration of reactive peptides measured in the three groups is illustrated in figure 4. By inspection, one may note that in general the samples from the nonsmoking control group yielded lower values and there was a smaller variation in the values of this group compared with the control smokers with no apparent disease and the individuals with COPD. In the nonsmoking control group, a substantial fraction of the samples contained no detectable peptides with an average value of 11.9 ng/ml and the maximal value was 42 ng/ml. In the normal smokers the average value was 53.5 ng/ml with a maximal value of 190 ng/ml, and in

TABLE 1

SUBJECT POPULATION STUDIED

	N	Age	Sex	Pack/Years	Mean $FEV_1/FVC\%$
Group I Nonsmoker	36	34 ± 15	22 M 14 F	0	80 ± 5
Group II Normal smoker	33	35 ± 14	13 M 20 F	20 ± 19	78 ± 4
Group III COPD	40	57 ± 17	28 M 12 F	49 ± 31	50 ± 16

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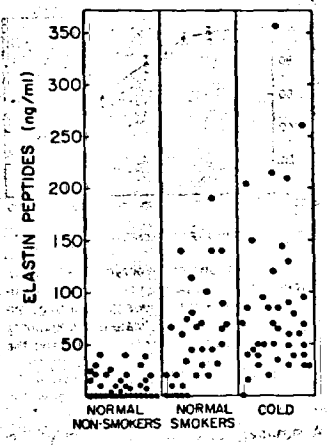

Fig. 4. Elastin peptides in human serums. Samples were assayed by the ELISA described in the text.

the COPD samples the average was 81.1 ng/ml with a maximum of 360 ng/ml.

Because of the disparity in the variance of the three groups, parametric statistics could not be employed; therefore, nonparametric statistical analyses were used. The data were first analyzed by the Kruskal-Wallis test (13). In this test all the measured values are ordered by rank and the ranks within the groups analyzed to determine whether the groups differ statistically from each other. The H value for the data illustrated in figure 4 was 46.3, a value indicating that all the groups are not identical at the $p < 0.001$ level. This test, which is a prerequisite for futher analysis, is not informative with respect to which groups differ from each other. It simply gives the probability of all groups being identical. To determine which groups differ from each other, they were analyzed by the Scheffé (14) multiple contrast test comparing pairs of groups. The results of this analysis are given in table 2. They show that both the smoking group and the COPD group have a significantly higher ($p < 0.001$) concentration of elastin-derived peptides than the control group. There also was a difference between the nonsymptomatic smokers and the COPD patients but this was significant only at the $p < 0.1$ level.

TABLE 2

ELASTIN PEPTIDES IN HUMAN SERUMS*

Group	Sample Number	$\bar{X}$ ng/ml	$\bar{R}$	$\bar{R}_i - \bar{R}_1$
1. Control	36	11.9	27.0	
2. Smokers	33	53.5	60.9	33.9†
3. COPD	40	81.1	75.4	48.4†

* Elastin-derived peptides in the sera were quantitated by the enzyme-linked immunosorbent assay described in the text.

† Significant at the $p = .001$ level. $\bar{X}$ is the average value for the elastin peptides in each of the groups. $\bar{R}$ is the average rank in each group. $\bar{R}_i - \bar{R}_1$ is the difference in average rank between either Group 2 or 3 and Group 1, the control group.

Discussion

The present studies represent the first attempt to utilize immunologic analysis of elastin-derived peptides as a means of distinguishing individuals with normal lungs from those who either presently have emphysema or are at risk of developing it. Whereas significant differences between smokers, COPD patients, and control subjects have been observed, for this test to be useful in individual cases it must be able to determine whether a given value is abnormal relative to control values in the population or whether it represents a significant change from previous measurements of a given individual. To answer these questions, and to correlate findings with physiologic measurements, chronologic studies on a larger number of individuals must be carried out in conjunction with concomitant pulmonary function tests.

Other investigators have used assays for elastin breakdown products to detect lung destruction in patients with COPD. Goldstein and colleagues (15) used a chemical assay for urinary desmosine, Harel and associates (16) used a radioimmunoassay for desmosine, and Darnule and coworkers (17) used a radioimmunoassay for elastin peptides. Although there is some disagreement regarding differences between normal smokers and normal nonsmokers, all investigators have found increased levels of elastin breakdown products in patients with COPD. It seems likely that these assays may be clinically useful tests of lung destruction after their role is fully documented in particular clinical situations.

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The Role of Oxidative Processes in Emphysema[1-3]

AARON JANOFF, HARVEY CARP, PHILLIPE LAURENT, and LINGA RAJU

SUMMARY Elastase/elastase inhibitor imbalance in the lung has been implicated in the pathogenesis of pulmonary emphysema. In light of this, it may be significant that the activity of two major elastase inhibitors, alpha 1-proteinase inhibitor (alpha 1-antitrypsin, α1Pi) and bronchial mucous proteinase inhibitor, can be decreased by oxidizing agents. The effect can be observed with ozone, substances present in cigarette smoke, and oxygen metabolites generated by lung macrophages as well as peroxidative systems released by other phagocytic cells. Thus α1Pi recovered from lung washings of cigarette smokers has only half the predicted normal activity per mg inhibitor and contains 4 moles of methionine sulfoxide (oxidized methionine) per mole of inactive inhibitor. By contrast, α1Pi purified from nonsmokers' lung washings is fully active and contains only native methionine. At the same time, lung washes from some smokers show significantly greater hydrolytic activity against a specific synthetic elastase substrate than do lung washes of nonsmokers. These findings suggest that some smokers may develop an acquired imbalance between elastase and elastase inhibitor in their lungs, favoring activity of the enzyme. In addition to the potential effect of cigarette smoking on lung elastase/elastase inhibitor balance, smoking also may interfere with elastin repair mechanisms. Specifically, acidic water-soluble gas phase components of cigarette smoke prevent synthesis of desmosine cross-links during elastinogenesis *in vitro*. This report will attempt to correlate the foregoing information on biochemical changes in the lung induced by cigarette smoking with the development of emphysema in the smoker.

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Introduction

The theme of this symposium is "Proteases and Antiproteases in the Lung," especially the potential importance of these substances in mediating various forms of lung injury. The subject has been reviewed elsewhere, particularly in connection with the pathogenesis of pulmonary emphysema (1-6). It is well known that oxidizing agents also can produce tissue damage; indeed both proteases and activated species of oxygen have been implicated as mediators of immunologic lung injury (7). This report attempts to link these two pathways of pulmonary connective tissue destruction because, in addition to their capacity to injure cells and intercellular matrix directly, oxidants can also perturb the homeostatic balance between proteases and their regulatory inhibitors (8, 9). Particular emphasis will be placed on the inactivation of alpha 1-proteinase inhibitor (the major regulator of neutrophil elastase in the lower respiratory tract of man (10)) by oxidants in cigarette smoke and in secretions of phagocytic cells. Several other actions of cigarette smoke, which may also contribute to connective tissue abnormalities in the lung, will be discussed. Possible biochemical links between pulmonary emphysema and cigarette smoking will be summarized and several questions raised for future study.

Mechanism of Elastase Inhibition By Alpha 1-Proteinase Inhibitor and the Effect of Oxidizing Agents

Alpha 1-proteinase inhibitor (alpha 1-antitrypsin, α1Pi) appears to act as a pseudosubstrate for its target proteases, and the site at which recognition and/or proteolysis occurs represents the active-site of the inhibitor (11). Structural studies have shown that the active site of α1Pi contains a methionine-serine peptide bond (12-14), although the possible presence of multiple active sites has not been ruled out (14, 15). Apparently this methionine-serine bond is recognized early in a series of reactions that ultimately leads to formation of extremely stable complexes between one mole of protease and one mole of inhibitor. The high stability of this complex may be based on covalent bonding between the protease's active center and the active site of the inhibitor, although the precise mechanism is not known. In any event, the inhibition of elastase by α1Pi depends on the integrity of a methionine-serine sequence in the active site of the inhibitor, and perhaps that of an additional methionine residue near the active site as well (12, 13, 16). In addition to methionine, the configuration of lysine (17) and tyrosine (18) residues in α1Pi may also affect its antiprotease activity.

Oxidation of α1Pi by chloramine-T or N-chlorosuccinimide, under conditions that favor the selective oxidation of methionine thioether groups, results in a loss of inhibitory activity (13, 16, 19-22). Structural studies have since confirmed that inactivation of α1Pi by chloramine-T or N-chlorosuccinimide is associated with the oxidation of two methionine residues in the inhibitor, one of which is present in the methionyl-seryl active site (13, 22). Oxidation of the active site methionine residue results in reduced interaction of the inhibitor with neutrophil and pancreatic elastase as well as trypsin (13, 20-22). Chymotrypsin-inhibitory capacity of α1Pi is relatively unaffected by oxidation, whereas elastase-inhibitory activity is most severely affected (13, 20-22).

Oxidation of the inhibitor reduces the rate of its association with neutrophil elastase by a factor of 2,000 compared with that of the native inhibitor (21). Any elastase-α1Pi complexes that may be formed appear to be less stable than normal (21). Similar findings have been obtained with synthetic elastase substrates containing methionine adjacent to the scissile bond. Oxidation of the methionine side-chain in these synthetic peptides also reduces the rate of their interaction with PMN elastase (19, 23). Suppression of the inhibitory capacity of α1Pi by oxidation also renders this protein susceptible to proteolytic digestion. After incubation of oxidized α1Pi with elastase, the inhibitor is partially degraded (21). In addition to methionine oxidation, a recent study has shown that specific oxidation of tyrosine residues in α1Pi can also inactivate its inhibitory function (18).

Besides α1Pi, a potent local inhibitor of neutrophil elastase and cathepsin G is found in bronchial secretions (24). The bronchial mucous inhibitor (BMPi) has been purified and shown to be a cationic protein with a molecular weight of 10 to 13,000 daltons (25, 26). Gadek and colleagues (10) reported undetectable levels of BMPi in bronchoalveolar lavage fluids of humans, and they and others (27) have suggested that the primary function of BMPi is to protect the upper airways (bronchi, trachea, and nasopharynx) against proteolytically-mediated injury. The active site structure of BMPi is not presently known; however, oxidizable residues also appear to be present close to the reactive center of this inhibitor (Fritz H, personal communication). A closely related,

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low molecular weight, acid-stable inhibitor in dog submandibularis glands contains a methionyl-aspartyl bond in its elastase and chymotrypsin-directed reactive center (28). As might be expected, BMPi can also be inactivated by chemical oxidants *in vitro* (29).

To assess the potential importance of these findings for the pathogenesis of pulmonary emphysema in humans, one need only note that when the activity of circulating α1Pi is depleted by treatment of experimental animals with a chemical oxidant, connective tissue changes suggestive of early stages of panacinar emphysema develop (30). The human respiratory tract is constantly exposed to various sources of oxidizing activity. In addition to oxygen itself, selected human populations are exposed to oxidizing environmental pollutants such as ozone, oxides of nitrogen and sulfur, and a host of oxidizing free-radicals present in cigarette smoke (see following). Moreover, phagocytic cells present in the lung (alveolar macrophages, neutrophils) generate copious amounts of hydrogen peroxide and other activated oxygen metabolites (see following), and this effect is increased by smoking. In the following section, some of these oxidants and their *in vitro* and *in vivo* effects on lung elastase-inhibitors will be discussed in further detail.

Oxidants Produced by Combustion of Cigarettes

Cigarette smoke displays a wide range of free-radical mediated activities including peroxidation and alkylation reactions both *in vitro* and *in vivo* (31). Aqueous solutions of cigarette smoke can oxidize cysteine sulfhydryl groups, a reaction that is blocked by the free-radical scavenger thymol (32). Cigarette smoke contains approximately 2×10^{15} free radicals per puff (33). Half of these appear to be polymeric phenoxy radicals related to melanin-like tobacco leaf pigments and are present in the particle fraction of cigarette smoke. They have a half-life of 10 hours on cigarette filters or in organic solvents after extraction of filters. An equal number of free radicals are found in the gas phase of cigarette smoke. These are mainly oxygen-centered radicals with half-lives of 10 min in the gas phase and 1 min in aqueous solution. In addition to the radicals produced during burning of cigarettes, cigarette smoke also may contain up to 800 ppm of nitrogen oxides (NO_x), especially NO. Besides their own free-radical properties, NO_x can react with olefins in the gas phase of cigarette smoke (34) or with peroxides (including, perhaps, the H_2O_2 produced by lung macrophages) to generate potent oxy-radicals such as hydroxyl radical (34, 35).

In Vitro *Inactivation of Antiproteases by Cigarette Smoke Solutions*

Freshly prepared aqueous solutions of unfractionated cigarette smoke, partly clarified by low-speed centrifugation, can suppress the elastase-inhibitory capacity of human serum (19). Immunoelectrophoresis of mixtures of aqueous smoke solution, human serum, and elastase show decreased elastase-α1Pi complexes and increased free, active protease. At least in our hands, smoke-treated α1Pi and control α1Pi are indistinguishable with respect to total quantity, electrophoretic mobility, and antigenic behavior of the inhibitors. An important finding is that free radical scavengers such as thymol or hydroquinone prevent the suppression of serum elastase-inhibition by cigarette smoke (19). In the presence of such agents, elastase-α1Pi complexes again can be detected following immunoelectrophoretic analysis of enzyme-smoke-serum reaction mixtures, and free enzyme is not seen. Also, treatment of serum with model oxidants under conditions that favor selective oxidation of methionine groups leads to a similar suppression of serum elastase inhibition (16, 19, 20). These observations suggest that oxidation reactions, perhaps mediated by smoke radicals of the type discussed in the preceding section, may be responsible for the observed results.

Similar findings have recently been obtained using ozone, in place of cigarette smoke, as a source of oxidizing activity (36). Ozone decreased the inhibition of trypsin, chymotrypsin, and elastase by human α1Pi, both in plasma and in solutions of the pure inhibitor. Total loss of pancreatic elastase inhibitory activity required 18 moles of ozone per mole of pure α1Pi. (A corresponding loss of the ability to inhibit human leukocyte elastase also was observed). Ozone-inactivated α1Pi was reported to contain four residues of methionine sulfoxide as well as oxidized tyrosine and tryptophan residues (36).

Inactivation of the bronchial mucous inhibitor (BMPi) *in vitro* by aqueous cigarette smoke solutions (29) or tar fractions (37) also has been demonstrated. Thin-layer gel-immunofiltration analysis of mixtures of aqueous smoke-treated BMPi and human leukocyte elastase revealed decreased elastase-BMPi complexes, increased uncomplexed BMPi, and increased free elastase (29). Phenolic antioxidants also prevented the suppression of the elastase-inhibiting-capacity of BMPi by aqueous cigarette smoke (29). In addition, treatment of BMPi with chemical oxidants caused a similar suppression of elastase-inhibition (29).

Oxidants Produced by Phagocyte Metabolism

The oxidizing systems of phagocytes, which participate in microbial-killing by these cells, have recently been reviewed in some detail (38, 39). According to present knowledge, the main features of oxygen metabolism in polymorphonuclear neutrophil leukocytes (PMN) and, with some modification, in monocytes and macrophages are as follows.

Upon phagocytosis or exposure to certain membrane-active agents, phagocytes undergo a "respiratory burst" characterized by increased oxygen consumption, increased production of hydrogen peroxide, and increased utilization of glucose via the hexose monophosphate shunt. The increase in oxygen consumption may be related to activation of a cyanide-insensitive, membrane-bound, NAD(P)H-dependent oxidoreductase that catalyzes the single electron reduction of molecular oxygen to the superoxide radical, as in equation (i):

$$2O_2 + NAD(P)H \rightarrow 2O_2^{\cdot} + NAD(P)^{+} + H^{+}.$$

The NAD(P) generated in this reaction stimulates the hexose monophosphate shunt because NAD(P) is a substrate in the first step of this metabolic pathway. In this first step, glucose-6-phosphate is converted to 6-phosphogluconate by glucose-6-phosphate dehydrogenase. At the same time, NAD(P) is converted to NAD(P)H, thus replenishing the substrate required for the membrane oxidase in equation (i). NAD(P)H generated by the shunt is also utilized by glutathione reductase. (The latter enzyme, together with glutathione peroxidase, functions to detoxify any excess hydrogen peroxide produced by PMN in the course of the "respiratory burst".)

The superoxide radical formed in equation (i) can react directly, or this radical can generate more powerful activated oxygen species (*) or oxygen free radicals (**) by the reactions shown in equations (ii) and (iii).

Equation (ii), dismutation reaction:

$$O_2^{\cdot} + O_2^{\cdot} + 2H^{+} \rightarrow H_2O_2^{*} + O_2 \text{ (or singlet } O_2?)^{*}$$

Equation (iii), Haber-Weiss type reaction, trace metal (lactoferrin (40)?) catalyzed:

$$O_2^{\cdot} + H_2O_2 \rightarrow OH^{\cdot **} + OH^{-} + O_2 \text{ (or singlet } O_2?)^{*}$$

Theoretically, these activated oxygen species and oxygen-free radicals could inactivate α1Pi within the phagocytic vacuole or in the immediate environment of the phagocytic cell.

In addition to the reaction shown in equation (iii), hydrogen peroxide can participate in other oxidative processes by serving as a cofactor for myeloperoxidase of PMN and monocytes. A second cofactor, usually a halide such as chloride or iodide anion, also is required. (Thyroid hormones can serve as a source of iodide for PMN.) In these myeloperoxidase-catalyzed reactions, hypohalous acids (e.g., HOCl) or free halogens (e.g., iodine) can be formed and these, in turn, are powerful oxidizing agents.

Activation of the membrane-oxidase that initiates the foregoing series of reactions (see equation i) appears to require a limited-proteolysis step mediated by a serine-

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enzyme with chymotrypsin-like activity (41). However, PMN and monocytes of individuals with chronic granulomatous disease appear incapable of activating the membrane-oxidase during phagocytosis or exposure to membrane-active agents. In these subjects, a normal "respiratory burst" does not accompany the stimulatory event. This condition is one of several in which phagocyte oxidative reactions are impaired, with serious consequences for host antimicrobial defense.

Enhanced Oxidant Production by Lung Macrophages of Cigarette Smokers

Chronic cigarette smoking not only increases the numbers of PMN (42) and macrophages (43) in the lung, but also activates these cells metabolically (43). Among the metabolic pathways that are stimulated in smokers' macrophages are those leading to production of superoxide radical and H_2O_2 (44). It therefore seems reasonable to propose that stimulated phagocytes in the lungs of cigarette smokers might be capable of generating oxidants that, in turn, could inactivate proteinase inhibitors in the environment of these cells. Evidence showing that stimulated phagocytes can indeed inactivate α1Pi and BMPi *in vitro* has been obtained in several laboratories; some of this evidence will now be summarized.

In Vitro *Inactivation of Antiproteases by Products of Oxygen Metabolism in Phagocytes*

We previously showed that human PMN, phagocytosing opsonized antigen-antibody complexes absorbed to the outer surface of dialysis membranes, produce diffusable species of activated oxygen capable of inactivating the elastase-inhibitory capacity (EIC) of whole serum or pure α1Pi on the other side of the membrane (45). In this system, serum EIC was partially protected by superoxide dismutase, catalase, or mannitol, suggesting that hydroxyl radical, formed by interaction of superoxide radical and hydrogen peroxide, might have been responsible for the observed inactivation. Sodium azide also partly protected EIC, implicating myeloperoxidase-mediated reactions as well. A cell-free superoxide generating system (xanthine + xanthine oxidase) also was shown to inactivate α1Pi in this system.

In related studies, Matheson and colleagues (46, 47) demonstrated that purified myeloperoxidase (MPO) in the presence of hydrogen peroxide and a halide ion could catalytically inactivate α1Pi *in vitro*, whereas two protease inhibitors more resistant to oxidants (α2 macroglobulin and α1-antichymotrypsin) were not affected by such treatment. These same investigators also directly showed that the active site methionine residue in α_1Pi, as well as an adjacent methionine residue, had been oxidized to the sulfoxide form by the MPO system (47).

More recently, we have extended our studies of phagocyte-mediated oxidative inactivation of α1Pi to include human mononuclear phagocytes (blood monocytes and alveolar macrophages), as well as PMN (48). These cells were activated by a selective membrane perturbing agent, phorbol myristate acetate (PMA), rather than by phagocytosis. Under these conditions, "respiratory burst" products were formed without bulk release of azurophil granule constituents. Such stimulated cells generated superoxide radical and suppressed elastase-inhibition by human serum. Scavengers of superoxide radical and H_2O_2 partly prevented this effect. Immunoelectrophoretic analysis of elastase reacted with serum that had been exposed to stimulated PMN showed decreased α1Pi-elastase complexes and free elastase. Treatment of phagocyte-inactivated serum with dithiothreitol (a reducing agent) resulted in significant recovery of inhibitory activity against elastase suggesting that, originally, α1Pi had been oxidatively inactivated. Moreover, PMN and monocytes from a patient with chronic granulomatous disease (which do not undergo a "respiratory burst"), as expected, failed to produce detectable levels of superoxide anion after stimulation with PMA, and these cells also failed to suppress serum elastase-inhibition.

Although PMA stimulates phagocyte respiration (including O_2^- and H_2O_2 production), this agent induces only minimal azurophil degranulation. However, because myeloperoxidase (MPO) is capable of catalytically inactivating α1Pi (one mole of purified MPO was shown to inactivate 250 moles of α1Pi per min, in a cell-free system (46)), the small quantities of this enzyme released following PMA stimulation could combine with H_2O_2 generated by the "respiratory burst" and extracellular halide and could participate in α1Pi-oxidation in our experiments. In support of this, we were able to demonstrate that azide and cyanide (both inhibitors of heme enzymes such as MPO) partly protected elastase-inhibiting capacity of serum against inactivation by the phagocytes. Furthermore, depletion of Cl^- from the medium also partly protected serum elastase inhibitory capacity from inactivation by stimulated PMN, suggesting that inactivation of α1Pi is partly dependent on a halide cofactor. Catalase, which decreases available H_2O_2, almost completely protected serum EIC from inactivation by PMA-stimulated cells.

In addition to the foregoing studies, Clark and coworkers (49) also reported that the MPO-system plays an important role in mediating oxidative inactivation of α1Pi by PMA-stimulated PMN, but they found a less significant inhibition by O_2^- and its derivative-activated oxygen species. Although mature human macrophages are believed not to contain myeloperoxidase, macrophages may contain other forms of peroxidase. Indeed, recent evidence suggests that a macrophage peroxidatic enzyme also may be able to inactivate α1Pi in a cell-free system (47).

The prediction that α1Pi may be inactivated by phagocyte-derived oxidizing systems *in vivo* has been borne out by the work of Wong and Travis (50), who demonstrated inactive α1Pi in synovial fluid obtained from inflamed joints and showed that the inactive inhibitor was in the oxidized state. A total of four methionine residues per molecule, including the active site methionine, were present as methionine sulfoxide. Other amino acids were unmodified, although a 17 amino acid peptide fragment was missing from the amino terminus of the inhibitor.*

The low molecular weight mucous inhibitor, human BMPi (that helps protect the mucus-lined airways against PMN elastase and cathepsin G), also has been shown to lose activity after *in vitro* exposure to myeloperoxidase, hydrogen peroxide, and chloride ion (29). Tsan and Chen (51) recently even demonstrated oxidation of free methionine by PMN and ascribed the effect to myeloperoxidase-mediated reactions and to singlet oxygen.

Macrophage H_2O_2 Also Could Act Synergistically with Oxidants in Cigarette Smoke to Inactivate Lung α1Pi

Pryor and coworkers (35) have shown that NO (and its derivative NO_2) can react with H_2O_2 to produce hydroxyl radical (a potent oxidizing agent) and other oxy-radicals, and have suggested that such reactions could take place in the lungs of smokers (34). Presumably, metabolically stimulated lung macrophages could serve as a source of H_2O_2 (see preceding section on enhancement of macrophage H_2O_2 production in cigarette smokers), whereas NO_x have been identified as cigarette combustion products. Oxy-radicals produced by interactions of H_2O_2 and No_x could contribute to the oxidative inactivation of lung α1Pi in the cigarette smoker (34). Evidence indirectly supporting this suggestion has been obtained by Cohen (personal communication),† who finds that aqueous smoke solutions from

* Since this article was submitted, Campbell and coworkers studied neutrophils in contact with ^{125}I-labeled fibronectin and reported that oxidants released from the stimulated cells did not protect secreted elastase against inhibition by α1Pi. (Campbell EJ, Senior RM, McDonald JA, Cox DL. Proteolysis by neutrophils. Relative importance of cell-substrate contact and oxidative inactivation of proteinase inhibitors in vitro. J Clin Invest 1982; 70:845–52.)

† Also, Cohen AB, James HL. Reduction of the elastase inhibitory capacity of alpha 1-antitrypsin by peroxides in cigarette smoke. An analysis of brands and filters. Am Rev Respir Dis 1982; 126:25–30.

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different brands of cigarettes inactivate α1Pi in direct proportion to their ability to oxidize o-dianisidine, *and that addition of H_2O_2 to the smoke solutions greatly augments both o-dianisidine oxidation and α1Pi inactivation*. Cohen also suggests that H_2O_2 produced by macrophages in the small airways of cigarette smokers could enhance the oxidative damage to lung α1Pi caused by constituents of inhaled cigarette smoke.

Thus multiple reaction pathways can be envisioned by which α1Pi could be oxidized and inactivated in the lungs of smokers. Direct action of oxidants in smoke is one possibility. Another is that the involved oxidants are produced by lung cells stimulated by smoke exposure. Still a third could involve oxidants generated by interactions between smoke components and products of cell metabolism. It now remains to present the evidence supporting the view that oxidative inactivation of α1Pi actually takes place *in vivo*.

In Vivo *Evidence for Inactivation of Lung Antiproteases by Cigarette Smoking*

Earlier, we found that brief inhalation exposure of rats to 3 or 6 puffs of cigarette smoke significantly decreased elastase inhibitory capacity per mg α1Pi in lung lavage fluid (52). Gadek and colleagues measured the inhibitory activity of α1Pi obtained from the lungs of smoking and nonsmoking humans and reported a nearly twofold reduction in the functional activity (versus pancreatic elastase) of this inhibitor in the lungs of cigarette smokers (53). Recently, we confirmed the fact that functional activity of α1Pi is significantly decreased (40%) in smokers' bronchoalveolar lavage fluids (BAL) compared with α1Pi derived from nonsmokers' BAL. In our study (54), the suppressant effect of smoking on α1Pi activity was shown using PMN elastase as test enzyme. Moreover, suppression of α1Pi was found to be reproducible when selected smokers and control individuals were lavaged on repeated occasions. Crossed antigen-antibody electrophoresis using antibodies directed against α1Pi and elastase confirmed that inactivation of α1Pi was responsible for the decrease in EIC of smoker's BAL. However, other workers (Stone and coworkers, Aspen Lung Conference, June 1982) failed to detect decreased levels of functional α1Pi in the lower respiratory tract of human smokers. A critical variable may be the length of elapsed time between last cigarette smoked and lung lavage in different laboratories.

Cigarette smoking also may be associated with decreased functional activity of bronchial mucous proteinase inhibitor (BMPi). Starting with tracheal aspirates obtained from patients undergoing elective surgery for non-pulmonary disorders, we partially purified BMPi from mucus collected from 15 smokers and 21 nonsmokers. Trypsin, chymotrypsin, and PMN elastase-inhibiting-capacities of the partly purified fractions were measured. In comparison to nonsmokers, smokers showed a 20% decrease in functional activity of BMPi per mg of inhibitor (measured by radial immunodiffusion against anti-BMPi antibody). This reduction was not related to patient age or sex (29).

Evidence that In Vivo *Inactivation of α1Pi in Cigarette Smokers is Due to Oxidation*

Using the animal model referred to in the preceding section (i.e., rats acutely exposed to cigarette smoke inhalation), we obtained indirect evidence suggesting that lung α1Pi inactivation *in vivo* is caused by oxidation of the inhibitor. We found that rats adapted to oxidant-injury by exposure to 2 ppm ozone for 4 h, one wk prior to cigarette smoke inhalation, had normal levels of α1Pi activity in their lung lavage fluids after smoking. Control, unadapted rats showed a 30% decrease in elastase-inhibiting-capacity per mg lung α1Pi after identical smoke exposure (52). In addition, when lavage fluids obtained from smoke-exposed rats were dialyzed against the reducing agent, sodium metabisulfite, there was a 75% recovery of normal elastase-inhibiting-capacity (52). The recovered inhibitory capacity was solely due to reactivated α1Pi, because antibody to rat α1Pi completely abolished the activity of sodium metabisulfite-treated samples. The reducing agent had no effect on the activity of the test enzyme.

In studies on human smokers (54), we obtained direct evidence to support the suggestion that lung α1Pi inactivation *in vivo* is caused by oxidation of the inhibitor. It was shown that partially inactivated α1Pi purified from smokers' bronchoalveolar lavage fluids (BAL) contained methionine sulfoxide, whereas fully active inhibitor purified from BAL or serum of nonsmokers did not. (Although we only detected changes in methionine, our conditions were designed to facilitate methionine sulfoxide quantitation and, conceivably, may have masked additional changes in other amino acid residues). Four moles of methionine sulfoxide were detected per mole of inactive α1Pi, which conforms closely to the value obtained by Wong and Travis (50) for α1Pi purified from rheumatoid synovial fluid. In the latter case, sequence analysis showed that the active-site methionine had been oxidized. Similar studies will be necessary to establish the location of the oxidized methionine residues present in smokers' lung fluid α1Pi.

The partially inactive α1Pi present in smokers' BAL could not be distinguished from the active α1Pi present in nonsmokers' BAL on the basis of immunoreactivity, electrophoretic mobility, or molecular weight. Thus, smokers' lung α1Pi appears not to be grossly denatured, inactivated by complex formation with granulocyte proteases (55), or significantly degraded by thiol-proteases (56), or by macrophage elastase (57). These latter agents alter the native electrophoretic mobility (55) or molecular weight of the inhibitor (56, 57). Alpha 1Pi inactivation by cigarette smoke *in vivo* also is not likely due to nonspecific denaturing effects of smoke on lung proteins (37) because functional activity of α-1-antichymotrypsin (a protease inhibitor that is resistant to inactivation by oxidation [58]) is unchanged in smokers' BAL.

Does Cigarette Smoke also Inactivate PMN Elastase?

In order for the oxidation hypothesis of smoker's emphysema, as put forward previously, to have merit, it is also necessary to show that cigarette smoke does not inactivate leukocyte elastase to the same extent as it does α1Pi. Although the enzyme is known to be more resistant than the inhibitor to inactivation by chemical and phagocyte-derived oxidants (20, 29, 45, 48), the relative rates of inactivation of these two proteins by *cigarette smoke* are unknown. Recently, Ohlsson and colleagues (37) reported that human granulocyte elastase is severely inactivated by dimethylsulfoxide solutions of the tar fraction of cigarette smoke, and Ejiofor and coworkers (59) reported partial inactivation of the enzyme by water-soluble, gas phase components of cigarette smoke. In the latter study, the enzyme was incubated with a relatively high concentration of smoke solution for prolonged time periods and the rate of its inactivation was measured. However, no effort was made to compare this rate with that of α1Pi-inactivation under identical incubation conditions.

We recently carried out an experiment (60) to compare the inactivation of human serum α1Pi and human granulocyte elastase by different concentrations and types of smoke extracts. Various amounts of aqueous solutions of gas phase cigarette smoke were incubated with pure human leukocyte elastase or with crude human leukocyte granule extract for 10 min at 37° C, and the effects on enzyme activity determined using a synthetic amide substrate. Simultaneously, equal concentrations of the same smoke solutions were incubated with 10% human serum under identical conditions, and the effects on serum inhibition of purified or crude leukocyte elastase were similarly measured.

In separate experiments, aqueous solutions of unfractionated cigarette smoke were incubated with pure leukocyte elastase or with serum for 30 min at 22° C. Ability of the smoke-treated enzyme to digest elastin and of the smoke-treated serum to inhibit elastin-digestion was then determined by measuring radial elastolysis in elastin-agar plates. Both experimental protocols clearly showed that serum elastase-inhibiting capacity is much more susceptible to inactivation by cigarette smoke than is leuko-

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cyte elastase when these proteins are incubated with water-soluble smoke extracts for relatively short times. For example, at concentrations of smoke that inactivated half the α1Pi (mimicking observed levels of α1Pi activity in smokers' lung wash (53, 54), only a 10% loss in PMN elastase activity could be measured. Similar results were obtained with both synthetic (amide) and natural (elastin) substrates, gas phase smoke and unfractionated whole smoke, and with pure enzyme and crude leukocyte granule extract (60).

An obviously important question is whether a short or a long incubation time *in vitro* best duplicates the conditions governing interactions among elastase, α1Pi and inhaled smoke *in vivo*. Our results at least suggest that should α1Pi and elastase in lung fluids be simultaneously exposed to inhaled smoke, half the antiprotease could become inactivated before the enzyme was significantly affected. This would potentiate the ability of elastase to attack its substrates in the lung before its own activity was suppressed by smoke products.

Other Effects of Cigarette Smoking on Lung Protease-Antiprotease Balance

Although not immediately relevant to the present discussion of oxidative processes in emphysema, at least brief mention should be made of the fact that cigarette smoking also causes increased numbers of PMN and macrophages to appear in the lung (42, 43), both of which cell types have the capacity to secrete elastase. The recruitment of PMN into smokers' lungs may involve prior activation of pulmonary macrophages by smoke components because stimulated alveolar macrophages can elaborate chemoattractants for PMN (42, 61–64). Further details can be found in earlier reviews of the subject (e.g., 5, 43).

The increased numbers of lung macrophages and PMN caused by smoking (as well as the inactivation of elastase inhibitors) leads to the prediction that increased elastase activity might be detectable in lung fluids of smokers. In preliminary studies by the authors (Janoff A, Raju L, Dearing R, unpublished observations), 7 out of 11 smokers showed elevations in PMN elastase activity of lung fluids compared with 11 nonsmoking control subjects (albeit involving very small amounts of enzyme (< 10 ng/mg lavage albumin)). The enzyme was measured in concentrated bronchoalveolar lavage effluent using the highly sensitive and specific elastase substrate, butyloxycarbonyl-l-alanyl-l-alanyl-l-prolyl-l-valyl-aminomethylcoumarin (65) (a generous gift of Dr. Morris Zimmerman; Merck Institute, Rahway, NJ). In one experiment, activity was completely inhibited by phenylmethanesulfonyl fluoride (10 mM) but only partly inhibited by ethylenediaminetetraacetate (10 mM), suggesting that the elastase might be a serine enzyme (PMN or monocyte-derived) rather than a metallo-enzyme (macrophage-derived) or that both enzymes were present. It may have been complexed to α2-macroglobulin because, in another experiment, activity of lavage was not significantly decreased by added serum.

Thus, lung fluids of smokers appear to contain some oxidized, inactivated α1Pi (see previously) and, in some cases, small amounts of elastase activity (but more than is found in nonsmokers' lungs). Taken together, these findings point to the possibility that protease-antiprotease balance in the lung may well be disturbed by such diverse effects of smoking as recruitment and stimulation of protease-secreting cells and interference with protease-inhibiting functions of α1Pi and BMPi. In the next section, an additional, newly discovered effect of smoking on elastin synthesis will be described, suggesting that even a third "chemical lesion" may result from smoking; one that could further exacerbate the development of emphysema in susceptible individuals.

Does Cigarette Smoking Exert an Inhibitory Effect on Elastin Repair?

Desmosine is a major cross-linking amino acid in elastin, and its formation is dependent upon the oxidative deamination of ϵ-amino groups of lysine residues in tropoelastin by the copper-dependent enzyme, lysyl oxidase. We recently (66) measured desmosine formation during elastinogenesis *in vitro*, using a cell-free system consisting of tropoelastin purified from chick embryo thoracic aorta or porcine aorta and lysyl oxidase purified from chick embryo or bovine lung. Desmosine formation was measured quantitatively using a specific, highly sensitive radioimmunoassay previously described (67). Desmosine synthesis could be demonstrated in this system with both homologous components (chick tropoelastin and chick lysyl oxidase) as well as with heterologous components (pig tropoelastin and bovine enzyme). Synthesis could be inhibited 80 to 90% in either system in the presence of a 1:20 dilution of aqueous extract of fresh cigarette smoke. Moreover, the inhibitory component(s) also could be demonstrated in the gas phase of filtered cigarette smoke and they behaved as negatively charged substance(s) during ion-exchange chromatography (66).

More recently, we also showed that water-soluble components of the gas phase of filtered cigarette smoke inhibit the first step in lysyl oxidase-catalyzed oxidative deamination of free ϵ-amino groups of elastin. The inhibitory effect of smoke was dose dependent. Because this step is also involved in formation of other elastin cross-links besides desmosine, the inhibitory action of cigarette smoke may have broad effects on elastin synthesis. The foregoing studies are described in further detail elsewhere (68).

Independently, Osman and associates (69) have simultaneously reported that tobacco smoke exposure retards elastin repair *in vivo*. Using hamsters in which experimental emphysema was first induced by a single intratracheal injection of pancreatic elastase, these investigators showed that pulmonary elastin content is regained within 1 wk after injury in animals not exposed to cigarette smoke. By contrast, elastase-injured "hamsters exposed to 6 to 7 puffs of cigarette smoke at hourly intervals for 8 h/day during the same repair period do not resynthesize elastin at the same rate." These workers injected ^{14}C-lysine during the repair phase after injury and found that "smoke exposure reduced ^{14}C-lysine incorporation into desmosine and isodesmosine by more than 40 percent" (69).

It has been suggested that elastin resynthesis may limit the degree of anatomic deformity in pulmonary emphysema (70), largely on the basis of evidence showing that lysyl oxidase inhibitors (such as β-aminoproprionitrile) greatly exacerbate the lesions induced by intrapulmonary elastase administration (70). Thus, the demonstration that elastin-synthesis is blocked by tobacco smoke components (66, 68, 69), may also help to explain the increased risk of pulmonary emphysema associated with cigarette smoking.

A Final Speculation: Inhibition of Transglutaminase by Cigarette Smoke—Potential Pathophysiological Effects on Lung

Cigarette smoke contains a complex array of chemicals, and smoke solutions may therefore modulate other pathways of lung metabolism besides those already mentioned in this report. For example, lung transglutaminases may be inhibited by cigarette smoke because transglutaminase in plasma (activated factor XIII of the clotting cascade) recently was found to be suppressed by smoke solutions (71).

Transglutaminases catalyze several acyl transfer reactions in which γ-carboxamide groups of peptide-bound glutamine residues serve as acyl donors. Epsilon-(γglutamyl) lysine cross-link formation (as in cross-linking of fibrin polymers during hemostasis) is perhaps the best recognized example (72). However, tissue transglutaminases may have several other functions. For example, a transglutaminase in macrophages has been implicated in regulating receptor-mediated endocytosis of α2-macroglobulin and other molecules (73). Might suppression of such an enzyme by cigarette smoke affect the endocytic activity of pulmonary macrophages? Again, fibroblast transglutaminases have been implicated in stabilization of a rigidly cross-linked cytoskeleton conducive to a nonproliferative state (74). Might inhibition of this enzyme function by inhaled cigarette smoke derange cytoskeletal architecture in some lung cells and increase the risk of lung cancer in smokers?

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Conclusions

I. Biochemical Links Between Cigarette Smoking and Pulmonary Emphysema—Current Status of the Hypothesis

Currently, three separate actions of cigarette smoking on lung metabolism have been recognized, which may act together to increase the risk of chronic obstructive lung disease in smokers. First, cigarette smoking may promote the oxidative inactivation of major elastase inhibitors in the lung (α1Pi and BMPi). Evidence was presented in this report implicating actions of smoke oxidants and of oxidants generated by metabolically-stimulated lung cells in smokers. Synergistic effects involving both forms of oxidizing activity also are possible. Second, smoking may simultaneously increase the elastase "burden" on the pulmonary connective tissue by activating the recruitment of macrophages and neutrophils to the lung. Third, newer evidence suggests a previously unsuspected effect of smoking, namely, inhibition of cross-link formation during resynthesis (repair) of elastin. These relationships are summarized in figure 1.

It seems reasonable to suggest that the distribution within the acinus of these three smoking-linked "biochemical lesions" (oxidation of α1Pi, recruitment of elastase-secreting cells, inhibition of elastin repair), might parallel the acinar distribution of inhaled smoke. Noxious components of cigarette smoke (oxidizing free radicals, cell-stimulating particulates, putative lysyl-oxidase inhibitors such as cyanide (75), or copper-chelators (76)) would be expected to have their greatest impact at the bifurcation of the terminal bronchiole and first-generation respiratory bronchioles (77). It is just in this centriacinar zone that the anatomic lesion of emphysema typically manifests itself in cigarette smokers. These relationships are depicted schematically in figure 2.

II. Questions for Future Study

Although the mechanisms proposed in this report, taken together, constitute an attractive hypothesis for the pathogenesis of emphysema in cigarette smokers, many questions still remain. Foremost among these is the need to explain the variable susceptibility among smokers to development of the disease. It seems safe to assume that there are other genetic and/or environmental risk factors affecting the outcome, in addition to those described previously.

One suggestion holds that antioxidants may play a key role. It has been suggested that ceruloplasmin, a major plasma antioxidant in man, can protect α1Pi against inactivation by myeloperoxidase (Taylor JC, personal communication). In addition, a newly discovered enzyme, methionine-sulfoxide reductase, is especially interesting in this regard. This enzyme was first purified from *E. coli*, but an analogous enzyme has since been demonstrated in mammalian tissues including lung and blood. The microbial enzyme can reduce methionine sulfoxide in proteins to native methionine (78) and is capable of partially reactivating chemically-oxidized α1Pi (79). Further studies of the mammalian methionine-sulfoxide reductase and of ceruloplasmin appear warranted because individual variation in the activity of these enzymes in lung or blood could conceivably exert a profound influence on pulmonary protease-antiprotease balance in smokers.

It is also worth noting that α1Pi function is depressed in bronchopulmonary secretions of infants exposed to hyperoxia, as part of current management protocols for infantile respiratory distress syndrome (IRDS) (80). Could protease-antiprotease imbalance, thus acquired, play a role in the development of bronchopulmonary dysplasia, a not infrequent, emphysema-like com-

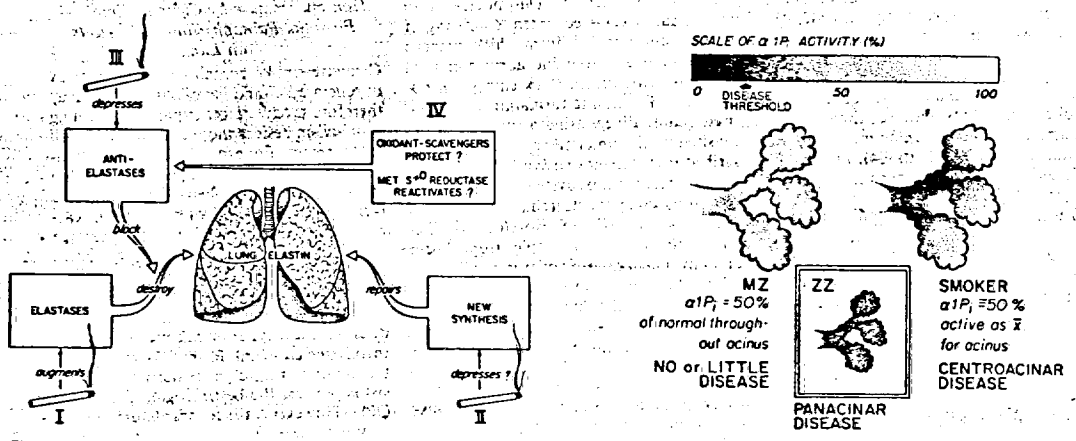

Fig. 1 (*left*). Proposed biochemical links between cigarette smoking and the pathogenesis of emphysema. (I) Smoking recruits monocytes, macrophages, and (through macrophage chemotactic factors) PMN to lung, elevating the connective tissue "burden" of elastolytic serine and metalloproteases. (III) At the same time, oxidants in smoke plus those produced by smoke-stimulated lung phagocytes (as well as oxidizing products of chemical interactions between these two) inactivate BMPi and α_1Pi, the latter representing the major antielastase "shield" of the respiratory units. (II) Other, unidentified water-soluble, gas-phase components of cigarette smoke (cyanide? copper-chelators?) inhibit lysyl oxidase-catalyzed oxidative deamination of ϵ-amino groups in tropoelastin and block formation of desmosine (and presumably other) cross-links during elastin synthesis, thus decreasing connective tissue repair. (IV) Anti-oxidants (ceruloplasmin? methionine-sulfoxide- reductase?) may protect or reactivate elastase-inhibitors, and other unidentified factors may modulate the chemical lesions induced in the lung by smoking so as to influence the risk of developing chronic obstructive lung disease. (Reproduced by permission from IM—Internal Medicine for the Specialist, Vol. 3, No. 3 March 1982.) Fig. 2 (*right*). Biochemical lesions induced by smoking are believed to parallel the distribution of inhaled smoke. In the diagram, only the inactivation of α_1Pi by oxidizing activity is shown, but the same reasoning can be extended to include the other 2 chemical lesions shown in figure 1. On the right-hand side of figure, the inhaled smoke constituents are shown as achieving their greatest concentration in the centriacinar zone (bifurcation of terminal bronchiole and first-generation respiratory bronchioles). Therefore, although the overall inactivation of α_1Pi measured in fluids recovered from the lower respiratory tract of smokers is about 50% (*see text*), the respiratory epithelium in the centriacinar structures may be more severely depleted of active elastase inhibitor. Thus, in smokers, active α_1Pi concentration in this zone may reach the disease threshold that is present throughout the acinus in homozygous α_1Pi deficiency (ZZ in the diagram). This could explain why centriacinar emphysema is most often associated with cigarette smoking whereas panacinar emphysema is more characteristic of ZZ disease. (MZ = heterozygous α_1Pi deficiency.) (Reproduced by permission from IM—Internal Medicine for the Specialist, Vol. 3, No. 3 March 1982.)

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plication of IRDS? Similarly, oxidative processes may disrupt protease-antiprotease balance in the lungs of adult patients treated with hyperoxia for acute respiratory distress syndrome (ARDS). Abnormal functional levels of PMN elastase and α_1Pi have been reported in some ARDS patients (see Cochrane's report in this symposium).

Future work also may extend the oxidation hypothesis to embrace lung injury associated with exposure to other environmental pollutants besides cigarette smoke. For example, oxidizing gases such as the oxides of sulfur and nitrogen, as well as ozone, are frequent environmental pollutants in urban areas and industrial sites, and one of these (ozone) has already been shown to inactivate α_1Pi *in vitro* (36). However, direct oxidant-injury to lung connective tissue, independent of protease-mediators, also must be considered in all of the foregoing. Indeed, oxidant cytotoxicity (81–83) and oxidant-mediated damage to intercellular macromolecules such as hyaluronic acid (84, 85) and collagen (86) are well established. Direct injury by oxidants has been proposed as the chief mechanism responsible for the interstitial pulmonary fibrosis that can be induced in experimental animals by intratracheal instillation of peroxidases together with an H_2O_2-generating system (87). Similarly, the role of direct oxidant-mediated injury needs to be explored in the pathogenesis of emphysema of cigarette smokers and individuals exposed to industrial pollutants.

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II. Prospects for Chemical Treatment of Emphysema

Introduction

GORDON L. SNIDER

Emphysema is defined as permanent enlargement of the respiratory air spaces of the lungs with destruction of their walls. All would agree that we do not know enough about the mechanisms of lung repair to have any hope of restoring emphysematous lung tissue to normal. But the development during the last two decades of the hypothesis that damage to the elastic fiber network of the lung underlies the development of emphysema, and that this damage is due to an imbalance of proteases and antiproteases in the lungs, clearly provides the opportunity for preventing emphysema. The evidence supporting this hypothesis is still fragmentary and largely indirect. The question obviously arises as to whether attempts to prevent emphysema by chemical treatment should be deferred until understanding of the basic pathogenetic mechanisms is more complete. There are many precedents for not doing so. Digitalis was used for treating heart failure and penicillin was successfully used for treating many infections long before the mechanisms of action of these drugs became clear. It seems entirely reasonable to attempt to manipulate the protease-antiprotease system in the lungs before we understand all its nuances.

Clinical trials requiring multi-center efforts are among the most expensive of all biologic research endeavors. In the first paper in this section, Hurd sets forth the consultative and administrative procedures for undertaking such research under the aegis of the Division of Lung Diseases (DLD) of the National Heart, Lung, and Blood Institute. Few other fund-granting agencies can afford to support such investigations.

The evidence supporting the protease-antiprotease hypothesis of emphysema is much more compelling in subjects with homozygous alpha-1-protease inhibitor deficiency than it is in genetically normal persons. However, this genetically determined form of emphysema comprises at most a few percent of the total population of individuals with emphysema. The problems of finding subjects with homozygous alpha-1-protease inhibitor deficiency before they have severe emphysema are formidable. Burrows, analyzing the results of the first two meetings of a DLD Working Group for Evaluation of Elastase Inhibitor Therapy in Pulmonary Emphysema, points out in the second paper the need for deciding when in the course of their disease such individuals should be studied. However, statistical projections indicate that 300 to 500 PiZ subjects with mild airways obstruction would need to be followed for a minimum of three years. Quite apart from the matter of expense, the enrollment of such a large number of PiZ subjects in a controlled trial of replacement therapy would be a difficult task, if indeed it is possible.

In the third paper of this section, Buist summarizes the results that were finally developed by the DLD workshops on the natural history of air-flow obstruction in groups of PiZ individuals collected in many institutions in the United States and in Sweden. As already noted, the chief purpose of these workshops was to determine the feasibility of a clinical trial of antiproteolytic therapy in PiZ individuals. Final analysis was limited to 105 subjects with a PiZ phenotype and with duration between first and last $FEV_{1.0}$ measurements of 12 or more months. Workshop participants fully recognized the limitations of retrospectively collected data. Furthermore, in some instances, affected persons presented as patients, and in other instances they were identified by screening of patients' relatives, blood bank samples, or persons participating in population studies. There is no assurance that the cases are representative of all PiZ subjects.

Within these limitations, the study provides information on changes over time of the $FEV_{1.0}$ in PiZ individuals that are now not available. It is not surprising that there was a high mortality but quite a low calculated mean rate of annual decline (45 ml/yr) in 52 PiZ subjects with $FEV_{1.0}$ values below 30% of predicted. Neither is it very surprising that 71 PiZ subjects with initial $FEV_{1.0}$ values between 30 and 65% of predicted had a mean rate of decline in $FEV_{1.0}$ of 107 ml/yr, a value almost twice the average rate of decline in patients (presumably non-PiZ) with ordinary COPD. The finding of 22 subjects with initial $FEV_{1.0}$ values greater than 65% of predicted who showed a relatively low mean rate of decline in $FEV_{1.0}$, 41 ml/yr, is of greater interest; these persons were mostly detected in population surveys. Thus, there is a population of PiZ individuals with moderate to severe air-flow obstruction who go through a period of relatively rapid functional decline, and these individuals would be suitable for entry into a clinical trial of the efficacy of antiprotease therapy. But those with very severe and those with very mild air-flow obstruction would not be suitable for such a trial. These data also suggest that advertising would be a more suitable way of finding PiZ subjects willing to volunteer for an antiprotease trial than population surveys of well people. The observations that some PiZ subjects with mild disease have a slow rate of functional deterioration is intriguing and seems worthy of further study.

In Glaser's paper, a method is described that should be readily adaptable to commercial-scale production for isolating alpha-1-protease inhibitor from Cohn Fraction-IV-1, a relatively unused side product in the worldwide production of albumin and immunoglobulin from human plasma. The method depends on the finding that alpha-1-protease inhibitor has a single, unusual disulfide bond that consists of a cysteine-containing region covalently linked to a free cysteine or glutathione via a disulfide bridge. The linkage can be broken by reductants without adversely affecting the stability or the protease-inhibitory activities of the protein. This finding permits the removal of contaminating proteins by salting out in the presence of strong reductants. Further purification of the product is carried out with DEAE-anion exchange chromatography. In order to minimize the high risk of contamination by hepatitis virus, a specifically developed pasteurization procedure is carried out.

Gadek and colleagues, using a different preparation of alpha-1-protease inhibitor, have demonstrated that blood and bronchoalveolar lavage levels of alpha-1-protease inhibitor can be raised to normal levels by weekly intravenous injections of the protein into persons with homozygous alpha-1-protease inhibitor deficiency. No side effects of treatment were encountered with 4 weekly treatments in 5 persons. Thus, the scene is set for the assumption by alpha-1-protease inhibitor of a major role in the preventive management of emphysema in alpha-1-protease inhibitor-deficient individuals. Needless to say, no private manufacturing company will undertake the commercial development of alpha-1-protease inhibitor without the assurance that a case-finding program and subsidization of treatment of alpha-1-protease inhibitor deficient individuals would follow.

Powers, in the final paper in this section, summarizes what is known of the chemical-

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ly active site of human neutrophil elastase. He discusses a number of potent reversible and irreversible inhibitors that have been developed for this enzyme. Several of these agents have been shown to be effective in preventing elastase-induced emphysema in animal models of the disease. However, little is known of the toxicity or pharmacology of these inhibitors and much work still needs to be done with them.

It is plain that other than for efforts bent toward decreasing the smoking of tobacco, the preventive management of emphysema is still a concept that is difficult and far off. But it is important to recognize that the approaches set forth in this section were not even dreamed of two decades ago. They are all the fruits of medical research that have cost but a tiny fraction of the sums of money that have been spent to care for the victims of emphysema. It seems clear that serious impediments, logistic, scientific and economic, stand in the way of a major initiative for the preventive management of emphysema in the near future. It can hardly be argued that, while these difficult decisions are being dealt with, government, industry, and private research organizations would do well to continue supporting basic research efforts in the pathogenesis of emphysema.

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What Are the Prospects for Getting the Current Theories Adequate Clinical Trials?[1,2]

SUZANNE S. HURD

Introduction

In October 1978, the Division of Lung Diseases, National Heart, Lung, and Blood Institute, convened a group of experts to consider the advisability and feasibility of developing and testing agents for the treatment of human pulmonary emphysema. The group was asked to evaluate the possibility of treating: (*1*) patients with a genetic deficiency of alpha-1-antitrypsin with isolated human alpha-1-antitrypsin or synthetic low molecular weight elastase inhibitors, and (*2*) treating more common varieties of emphysema with the elastase inhibitors. Based on available data, the conferees agreed in principle that it might be possible to treat emphysema, but they identified several formidable problems that would have to be solved before clinical trials of these therapies could be undertaken (*1*).

A proposed plan to meet these goals was divided into three phases. *Phase 1*: (*1*) Determine the natural history of emphysema in patients with phenotype PiZZ for alpha-1-antitrypsin. (*2*) Develop methods for isolation of alpha-1-antitrypsin. (*3*) Set up a means of testing the safety of elastase inhibitors. (*4*) Encourage development of new elastase inhibitors. (*5*) Encourage the development of biochemical means of detecting lung destruction in patients with emphysema. *Phase 2*: (*1*) Design the study of therapy of patients with PiZZ phenotypes and identify and enroll patients. (*2*) Produce therapeutic amounts of alpha-1-antitrypsin, prove its safety in animals and people, and determine the clinical usefulness in patients. (*3*) For common emphysema, produce therapeutic amounts of elastase inhibitors, prove safety in animals and people, and determine the clinical usefulness in patients. At phase 3, clinical trials would be conducted.

Many steps have been taken to meet the goal of improved management of emphysema, as evidenced from other presentations in this symposium. Although clinical investigations to demonstrate feasibility of alpha-1-antitrypsin replacement therapy in PiZZ subjects have been reported (*2*), clinical trials to provide the definitive validation step in testing the efficacy of this treatment regimen before it is introduced into practice have not yet been initiated. What are the prospects for getting the current theories relating to the treatment of pulmonary emphysema adequate clinical trials?

The Clinical Trial

The National Heart, Lung, and Blood Institute supports a large number of basic, clinical, and applied research projects that have as their objective understanding the etiology and pathogenetic mechanisms involved in disease processes. However, translation of results from the basic and clinical scientist into specific application for diagnosis and treatment of disease is an important step that must be taken. The clinical trial represents a mechanism whereby therapeutic modalities, among others, can be tested and validated before introduction into the health care system. A clinical trial may also be undertaken in order to determine which of several alternative treatments already in use are most effective.

Clinical trials have been defined (*3*) as cohort studies in which treatment is initiated specifically for evaluation and not just during the care or observation of patients. Such trials are further classified into uncontrolled trials, in which there is no concurrent comparison group; nonrandom controlled trials, in which concurrent comparison groups are allocated by means of some nonrandom process; and randomized controlled trials, in which subjects are randomly allocated into treatment and control groups. Depending on the hypothesis to be tested and the endpoint of the study, a clinical trial may be undertaken in a single center with just a few subjects or it may require participation of several centers or clinics to engage in a collaborative effort involving many subjects.

The steps to initiate a clinical study require the concerted effort of basic scientists and clinicians who must be confident that the scientific and medical basis of the proposed study is valid. If new drugs are to be tested, clearance procedures with the Federal Food and Drug Administration must be initiated. If a large amount of specific biomaterials are required, procedures for large scale production must be available and a process initiated to prepare sufficient amounts for testing purposes. Financial resources must be available to assure long-term commitment to participants in a clinical study. At a time when there are more demands on fewer Federal funds for research, the decision to implement a clinical trial through the NIH has become more critical. These trials are often expensive to undertake because of the need to involve multiple clinical centers over a long period of time to enroll sufficient numbers of subjects and follow them until the therapy can be effectively evaluated.

Steps in Initiation of a Clinical Trial: The Research Grant

The National Heart, Lung, and Blood Institute has provided financial support for

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many clinical studies through several different mechanisms. A single investigator or a group of investigators may choose to develop and design a clinical research investigation and submit a regular research grant. If adequate numbers of subjects are available to test the hypothesis and the necessary biostatistical skills are available, a single center trial is feasible. However, if multiple institutions are required in order to enroll adequate numbers of subjects or to minimize influence of geography, climate, or lifestyle, a group of investigators could decide to develop a common study protocol with biostatistical expertise included either in one of the cooperating clinical centers or as a separate unit. This later approach is called an "investigator-initiated multicenter clinical trial" (4). A research grant application for the support of a clinical investigation, whether single-center or multi-center, is reviewed for scientific merit through the regular NIH study section mechanism and competes for funding available through the research grant program.

The Research Contract

Another mechanism for support of a clinical trial is through a contract program initiated by the National Heart, Lung, and Blood Institute. In this case, specific funds are set aside in the Institute's budget to support the investigation. Because support for clinical trials, particularly those concerning preventive regimens, can be expensive, a specialized decision process has been developed by the Institute that has been a useful aid for the formulation, design, conduct, analysis, and dissemination of the results of clinical trials (5). The process includes major decision points at which the Institute commits resources to plan a trial, to conduct a trial, and to terminate the trial. At each decision point, the program is subjected to a series of reviews—peer review, Division advisory group review, Institute review, and Institute Advisory Council review. The reviews center on four basic factors: the state of the science, the feasibility of the proposed trial in terms of the likelihood of the results and the resources required, the potential impact of the trial, and ethical considerations.

Of particular importance to the conduct of a clinical trial is a Data and Safety Monitoring Committee, a committee charged with overseeing the ongoing trial results. The members keep careful watch for signs of toxicity from the intervention as well as a continual analysis of the efficacy of the treatment or preventive regimen. The committee will recommend to the Institute and ultimately the trial's steering committee that the study be terminated if intervention toxicity is noted or if the major question appears to be answered.

Where Do We Stand?

A significant investment of public and private funds has been made for studies of the protease-antiprotease theory of development of pulmonary emphysema. Important contributions to our understanding of the complex pathogenetic mechanisms have been reported. However, many questions remain to be answered before a large-scale clinical trial on treatment of emphysema would be endorsed by the scientific community, the Institute, and its advisors. For example, is there a consensus on the product to be tested? Although the homozygous PiZZ individual is the likely subject, at what point should intervention be considered? Have sufficient PiZZ individuals been identified for participation in a clinical trial or would massive screening programs first have to be undertaken? Will the low number of PiZZ subjects at any given clinical center require participation by such a large number of centers that the financial expense of conducting a trial would be prohibitive?

The NHLBI will continue to encourage and support as many meritorious research grant projects as possible in this important area. Workshops will be held to continue the planning process toward developing a clinical protocol for the testing of products that may be effective in the prevention or management of pulmonary emphysema. In all steps, we will continue to involve the scientific community through the Institute's advisory committees to evaluate the scientific information as it becomes available and to share in the complex decisions that must be made before biomedical resources are committed to initiate a clinical trial to test new agents for treatment of human pulmonary emphysema in a controlled clinical investigation.

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A Clinical Trial of Efficacy of Antiproteolytic Therapy: Can It Be Done?[1,2]

BENJAMIN BURROWS

In recognition of the growing interest in the development of antiproteolytic therapies for emphysema, the Lung Division of the National Heart and Blood Institute convened, in October 1978, a Working Group for Evaluation of Elastase Inhibitor Therapy in Pulmonary Emphysema. (The membership of this Working Group is indicated at the end of this paper.) Several conclusions were reached and recommendations made at this meeting. Most had to do with the development of improved antiproteolytic materials and studies of their pharmacology. The development of chemical markers of lung destruction in emphysema was also considered a high priority.

In regard to possible clinical trials of therapies that looked promising, it was recommended that any initial trial should be carried out on alpha-1-antitrypsin deficient PiZ subjects, because the emphysema of such subjects is clearly related to an antiprotease deficiency and should be more likely prevented or ameliorated by replacement therapy than in patients without demonstrable antielastase deficiency. However, it was recognized that little was known about the natural history of PiZ disease and that if this was as variable as that of "ordinary" chronic obstructive pulmonary disease (COPD), very large numbers of subjects would need to be followed for a prolonged period to demonstrate significant beneficial effects on progression of disease or on mortality. It was suggested that much might be learned by examining data already available in the United States and other countries regarding mortality and rates of functional decline in PiZ subjects. It was recommended that a workshop be convened to collect, discuss, and interpret the compiled information.

A Workshop on the Natural History of the PiZ Subjects was subsequently convened in March 1980 under the auspices of the Lung Division. Participants were invited to bring with them as many examples of PiZ disease as possible. The purpose of this report is to summarize the discussions at that workshop and to explain some of its conclusions.

Ideally, one might wish to carry out a trial of the ability of antiprotease administration to prevent the emergence of disease in asymptomatic PiZ subjects. Unfortunately, the proportion of PiZ subjects who develop emphysema is unknown, and the age of onset of frank disease appears to be quite variable. Thus there would be no way to determine sample sizes needed to determine the efficacy of therapy; very large numbers of subjects would probably be required. Furthermore, recruitment of large numbers of asymptomatic PiZ subjects would present a serious logistic problem. For this reason, it was felt that primary prevention efforts to demonstrate efficacy of therapy would be impractical, at least as a first step.

The possibility of using late stage PiZ emphysema cases was next considered, using mortality as an outcome. The fact that mortality is a clearly identifiable end point is a clear advantage in such a study. Also, by identifying patients with an expected high mortality, significant differences might be noted within a few years, as was the case in the Nocturnal Oxygen Therapy Trial (1). In that study, significant differences in mortality were noted in a sample of a few hundred COPD patients followed for less than three years.

Despite these potential advantages, the group believed that there were overriding disadvantages in studying preterminal illness. First, the late stages of the disease may be "self-perpetuating" and too late for antiprotease to be effective. Also, the relationship of mortality to the stage of the disease is unknown in untreated PiZ emphysema. If it resembles ordinary COPD, initial functional levels, age, and evidences of cardiac disease become critical variables (2) that would need to be controlled. Furthermore, a high rate of intercurrent diseases might greatly influence results, especially in older subjects.

The conclusion of the group was that the best type of clinical trial would involve PiZ subjects already showing definite but relatively mild airways obstruction. This would provide an opportunity to arrest progression of the emphysematous process at a relatively early stage when rates of progression might be discernibly altered. Even such a study is fraught with problems, however.

Because mortality rates would be expected to be relatively low in these patients, the rate of decline in function would be the only practical end point of the study. Rates of decline are relatively slow and show wide variability in subjects with "ordinary" COPD. Whether the rates of decline in ventilatory function are as low and as variable in PiZ emphysema is unknown. If they are similar to those in ordinary COPD, enormous numbers of experimental and control subjects might be needed to show significant differences between treated and untreated groups.

The magnitude of the variability in rates of decline is exemplified in table 1, representing data from a study of 200 COPD patients enrolled in an emphysema study in Chicago more than 20 years ago (3). The variability in rates of functional loss is very high when there are only a few years of follow-up, but variability decreases with time of study. Obtaining more frequent observations tends to reduce the standard deviations of calculated regression slopes, but only to a limited extent, because the inherent fluctuations in FEV_1 measurements may actually exceed the calculated rates of decline for the first few years of follow-up. Because the sample size needed to demonstrate significant differences between experimental and control groups depends not only on the reduction in mean decline in the experimental group but on the standard deviations around this mean decline, enormous sample sizes would be required with less than three years of follow-up.

TABLE 1

RATES OF DECLINES IN FEV, AND THEIR STANDARD DEVIATIONS (S.D.) IN UNSELECTED COPD SUBJECTS VERSUS LENGTH OF FOLLOW-UP (CHICAGO STUDY)

Duration (yr)	Mean Decline (ml)	S.D. (ml)
1	−83	218
2	−69	153
3	−62	97
4	−57	83
5	−56	85
6	−58	84

Based on the assumption that PiZ emphysema would behave like "ordinary" COPD, Dr. Margaret Wu, mathematical statistician for the Heart, Lung, and Blood Institute, calculated sample sizes (combined equal-sized experimental and control groups) needed to show significant differences ($p < 0.05$) between the rates of decline of treated and untreated subjects. As seen in table 2, these depend both on the proposed duration of follow-up and on the anticipated magnitude of the effect. It was believed that it would be unrealistic to expect more than a 50% rate of decline because this would give the experimental group a rate of fall close to that of the normal population. As can be seen from the table, 300 to 500 subjects would be required for a therapeutic trial with a minimum follow-up of three years. Many of us believe that enrollment of

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TABLE 2

MINIMAL SAMPLE SIZES OF PiZ DISEASE TO SHOW A SIGNIFICANT DIFFERENCE BETWEEN TREATED AND CONTROLS IF TREATED SHOWED A 40% OR 50% REDUCTION IN DECLINE IN FEV,*

Years follow	Number if 50%	Number if 40%
3	336	524
5	316	494
7	290	454

* Assuming as much variance as in unselected COPD.

this number of PiZ subjects might be difficult indeed.

All of this presumes that PiZ disease is as variable as ordinary emphysema. If it were more stereotypic in its behavior, the numbers might be greatly reduced. To see how stereotypic PiZ disease appeared to be, participants in the Workshop were asked to examine retrospectively their data on the characteristics and courses of their PiZ patients. In this way, a large number of anecdotes were assembled and discussed at the PiZ workshop mentioned above. There was almost universal agreement that a minority of PiZ patients had the typical uncomplicated emphysema so often described as characteristic of PiZ disease. Also, there appeared to be wide variability in the course and prognosis of these patients, similar to that seen in "ordinary COPD." However, it was felt that more complete data were needed before final conclusions could be reached. Various physicians around the country and in Sweden were asked to complete a standardized form describing the characteristics and courses of their PiZ patients and submit these to Dr. Margaret Wu in Bethesda. These data have been checked and are being analyzed. Hopefully, they will indicate a lesser variability in rate of decline than was suggested by a less formal look at preliminary data.

At the time of this symposium, it is difficult to say whether a clinical trial of antiproteases in PiZ disease is feasible or not. This will depend on some of the statistical considerations already discussed. It will also depend, however, on the nature, expense, and difficulty of applying any proposed antiprotease program. It is clear, however, that any trial of antiprotease therapy will require a large multi-center effort with screening of all patients with persistent airflow limitation for Pi phenotype if adequate numbers of patients are to be assembled for a meaningful clinical trial.

Membership of the Working Group for Evaluation of Elastase Inhibitor Therapy in Pulmonary Emphysema: Allen B. Cohen, M.D., Ph.D., Chairman; Benjamin Burrows, M.D.; Ronald Crystal, M.D.; Graham Jamieson, Ph.D.; Aaron Janoff, Ph.D.; Alan Johnson, M.D.; Philip Kimbel, M.D.; James C. Powers, Ph.D.; Richard C. Talamo, M.D.; Gerard M. Turino, M.D.; Margaret Wu, Ph.D.

Participants in the "Workshop on the Natural History of The PiZ Subject" meeting in March 1980 included: Robert C. Allen, Ph.D.; Leo F. Black, M.D.; Sonia Buist, M.D.; Benjamin Burrows, M.D.; Bernice H. Cohen, Ph.D.; Allen B. Cohen, M.D., Ph.D.; Ronald Crystal, M.D.; Robert J. Fallat, M.D.; Morton Galdston, M.D.; Philip Kimbel, M.D.; Christer Larsson, M.D.; Stewart A. Lonky, M.D.; Charles Mittman, M.D.; John A. Pierce, M.D.; Gerard M. Turino, M.D.; and Margaret Wu, Ph.D.

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The Natural History of Air-flow Obstruction in PiZ Emphysema[1–4]

REPORT OF AN NHLBI WORKSHOP

Prepared by A. Sonia Buist, Benjamin Burrows, Sten Eriksson, Charles Mittman, and Margaret Wu[4]

Introduction

The association between severe alpha-1-antitrypsin (AAT) deficiency and pulmonary emphysema was first described by Eriksson in the early 1960s (1, 2). These initial reports created considerable interest and provided the first real evidence that protease inhibitor factors in serum play an important part in maintaining the integrity of the lung parenchyma. A large body of evidence now supports the theory that emphysematous destruction results from an imbalance between proteolysis and antiproteolysis within the lung. However, little is known about the natural history of patients with emphysema resulting from severe AAT deficiency.

The data reported here were gathered by a group of investigators who participated in a workshop convened by the Division of Lung Diseases, National Heart, Lung and Blood Institute (NHLBI). The primary purpose of the workshop was to determine the feasibility of a clinical trial of antiproteolytic replacement therapy in PiZ subjects, should such therapy become available in the near future. In order to address this question, it was necessary first to collect information on the natural history of airflow obstruction in PiZ disease.

Retrospective data on PiZ individuals, which had been collected at many institutions in the United States, were reviewed. Also, comparable data were obtained for PiZ subjects seen in Sweden. We examined the natural history of the airflow obstruction, as reflected in the rate of decline of the 1-s forced expiratory volume (FEV_1). There was good agreement between results in American and Swedish subjects, giving us some confidence that the rates of decline of lung function observed may be reasonably representative of the rates of decline of function that occur in the subset of PiZ individuals who develop frank airflow limitation.

Methods

Information was provided by workshop members on 298 individuals living or having lived in the United States and who were known to have an AAT deficiency state. Sources of data included: (*1*) patients with lung disease, (*2*) relatives of such patients,

[1] A report of a workshop on the natural history of PiZ emphysema, sponsored by the Lung Division of the National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland. The workshop met on two occasions, March 19–20, 1980 and November 9, 1981.

[2] Workshop members: Robert C. Allen, Ph.D.; Leo F. Black, M.D.; A. Sonia Buist, M.D.; Benjamin Burrows, M.D.; Bernice H. Cohen, Ph.D.; Allen B. Cohen, M.D.; Sten Eriksson, M.D.; Robert J. Fallat, M.D.; Morton Galdston, M.D.; Stewart A. Lonky, M.D.; Harold A. Menkes, M.D.; Charles Mittman, M.D.; John A. Pierce, M.D.; Gerard M. Turino, M.D., and Margaret Wu, Ph.D.

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[4] All statistical analyses were carried out by Dr. M. Wu, mathematical statistician for the National Heart, Lung and Blood Institute.

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TABLE 1

FINDINGS IN U.S. AND SWEDISH PiZ SUBJECTS WITH INITIAL FEV_1s BETWEEN 30- AND 65% OF PREDICTED

	U.S.	Swedish
Number of subjects	30	41
Mean age (SD), yr	45 (9)	47 (8)
Annual change in FEV_1, mean (SD), L	−0.111 (0.102)	−0.104 (0.094)
Initial FEV_1, % predicted, mean (SD), %	41.9 (10.7)	43.3 (9.6)
Months of follow-up,* mean (SD)	62 (36)	74 (47)
Percent males	50%	63%
Never smokers	3%	17%
Ex-smokers	80%	59%

* Number of months between first and last FEV_1 measurement.

(3) persons identified by blood bank screening, and (4) subjects identified in the course of population studies. For each subject, all known pulmonary function data were provided plus trypsin inhibitory capacity measurements, Pi phenotypes, age, height, and either date of death or daté last known alive. Initial and follow-up spirometric values were available on just over half of the subjects. After eliminating those who had not been shown to have a PiZ phenotype and those with duration between the reported initial and last FEV_1 measurements less than 12 months, 105 subjects from the United States remained for the study. Data collected on PiZ subjects seen in Sweden were provided by Dr. S. Eriksson. There were 41 Swedish patients meeting all the criteria mentioned above.

For the subjects included in the study, the number of FEV_1 measurements reported ranged from 2 to 12 (mean of 4.1) in the U.S. group and from 2 to 10 (mean of 3.8) from the Swedish group. The duration between the reported initial and last FEV_1s ranged from 13 to 149 months for the U.S. group and from 12 to 181 months for the Swedish group.

After preliminary analyses of the data on these subjects, it was decided that the most important group in terms of a possible therapeutic trial were those 30 U.S. subjects who, in addition to fulfilling the above criteria, were also less than 65 yr of age when first diagnosed and had an initial FEV_1 value between 30- and 65% of predicted (3). The reasons for concentrating on this group will be discussed below.

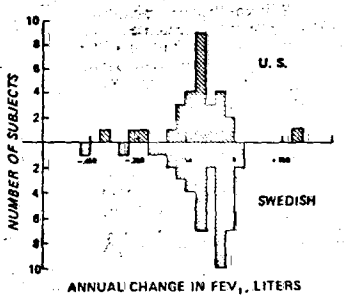

Fig. 1. Rate of change in 1-s forced expiratory volume (FEV_1) in liters/year for 30 U.S. subjects and 41 Swedish subjects with homozygous α_1-antitrypsin deficiency, PiZ phenotype. All subjects were under age 65 and had an FEV, between 30- and 65% of predicted (3).

Linear regression analysis was carried out separately for each individual to determine the annual rate of change in FEV_1.[4] From these analyses, group mean rates of decline were calculated and the standard deviations of these rates determined. Because our method of selecting subgroups for analysis could lead to some regression toward the mean, analyses were also carried out after deleting the initial FEV_1 levels, using only subjects with at least two subsequent follow-up studies. This analysis resulted in a 10% reduction in the mean rates of decline from those that included the initial data point. However, this method of analysis did reduce the number of subjects available for study. Consequently, because regression toward the mean did not appear to be affecting our results significantly, data reported here are based on analysis using all available FEV_1 values.

Results

Preliminary analyses showed that there was a small number of healthy young adults who were primarily detected in population surveys and who had normal or near normal FEV_1 values with very little, if any, decline in FEV_1 on follow-up. For example, there were 22 PiZ subjects in the U.S. group with an initial FEV_1 greater than 65% of predicted. This group had a mean age of 38 ± 12 years and a mean annual decline in FEV_1 of 42 ± 52 ml. It was also apparent that there was a high mortality but a quite low calculated rate of decline in subjects with FEV_1s below 30% of predicted. The 52 PiZ subjects in this group had a mean age of 46 ± 9 years and a mean annual decline of FEV_1 of 45 ± 8 ml.

Quite different rates of decline were noted in the 30 U.S. subjects under age 65 with FEV_1s in the 30–65% of predicted range. As seen in table 1, these subjects showed an annual decline in FEV_1 of 111 ± 102 ml. The majority of these patients came to the attention of investigators as patients. Data on 41 Swedish subjects showed very similar mean rates of decline and standard deviations. As also shown in table 1, the U.S. and Swedish subjects were very similar in regard to other characteristics, such as mean percent predicted FEV_1 on entry, sex distribution, and smoking habits. The distribution of rates of decline in the two series is shown in figure 1. Again, results are strikingly similar.

Discussion

Participants in the workshop fully recognize the limitations in data accumulated in the manner described. There is no assurance that the cases assembled are in any way representative of all PiZ subjects in the population. Indeed, as noted above, it is clear that not all persons with the PiZ phenotype develop overt disease. It is possible that their PiZ status was discovered only because of some unusual characteristic of their disease. In many instances, it was discovered because they developed overt disease. For this reason, direct comparisons with series of "ordinary COPD" subjects may not be totally valid. However, the rates of decline in FEV_1 observed in subjects with clinically significant but not end stage PiZ disease are nearly twice the average rates of decline reported in "ordinary COPD" (4).

In contrast, PiZ subjects who do not yet show a clinically significant degree of airways obstruction show much lower rates of decline. This may be explained in one of two ways. It is possible that the functional consequences of gradually progressive anatomic changes in the lungs of PiZ patients become manifest only after the disease is quite extensive. Functional changes may then progress relatively rapidly. This is compatible with observations of mild emphysematous changes in the lungs of PiZ patients dying of hepatitis at a young age (5). Alternatively, it is possible that not all PiZ subjects develop significant pulmonary disease and, unless one excludes such subjects, mean rates of functional decline may appear quite low.

At the other end of the spectrum, patients with very severe initial functional impairment cannot demonstrate high rates of decline because such declines would be incompatible with life. Thus, the low rates of decline in subjects with an initial FEV_1s below 30% of predicted are likely to represent a "survivor effect."

From the data presented, it would appear that PiZ subjects who develop clinically significant airflow limitation go through a phase of their disease in which there is a relatively high rate of functional decline. This is encouraging in regard to the feasibility of a trial of antiprotease therapy. It suggests that beneficial effects of such therapy could be demonstrated by showing a reduction in the rate of loss of function in subjects properly selected for such a clinical trial.

Acknowledgment

We gratefully acknowledge the help of the Division of Lung Disease staff in the preparation of this report, in particular, Eric R. Jurrus, Ph.D, Hannah H. Peavy, M.D., and Richard J. Sohn, Ph.D.

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Experience with Replacement Therapy in the Destructive Lung Disease Associated with Severe Alpha-1-Antitrypsin Deficiency[1,2]

JAMES E. GADEK and RONALD G. CRYSTAL

Studies over the past 20 years have fostered a dramatic improvement in our understanding of the disease mechanisms underlying pulmonary emphysema. It is fitting that the "experiment in nature" that provided the foundation for the protease theory of emphysema should provide the initial opportunity to translate these pathogenetic insights into specific therapy (1). In the interim since the landmark observation by Laurell and Erickkson (2), studies performed in a number of laboratories have served to demonstrate the relevance of serum alpha-1-antitrypsin deficiency to the destruction of alveolar structures. Most importantly, it is now recognized that: (*1*) neutrophil elastase is inhibited by alpha-1-antitrypsin (3); (*2*) experimental destructive lung disease can be produced by human neutrophil elastase (4, 5); (*3*) the risk of developing destructive lung disease is directly related to the severity of the alpha-1-antitrypsin serum deficiency (i.e., the PiZ phenotype of alpha-1-antitrypsin is the phenotype most commonly associated with premature emphysema and, with rare exception, the PiZ phenotype is also associated with the lowest serum levels) (6); (*4*) individuals with the PiZ form of alpha-1-antitrypsin deficiency are devoid of effective inhibitory activity against neutrophil elastase at the level of their alveolar structures (7); and (*5*) PiZ patients with destructive lung disease have a chronic influx of neutrophils (8). Together, these observations strongly support the concept that the lung destruction associated with alpha-1-antitrypsin deficiency results from the unimpeded action of neutrophil elastase on the alveolar structures.

In this context, it is reasonable to propose that, like other serum protein deficiencies such as inherited hypogammaglobulinemia (9), hemophilia (10), and hereditary angioedema (11), alpha-1-antitrypsin deficiency could be corrected by parenteral replacement therapy. However, there are several formidable obstacles that must be overcome before alpha-1-antitrypsin therapy becomes a reality.

Most importantly, the expression of destructive lung disease in alpha-1-antitrypsin deficiency is an insidious, progressive loss of lung function that culminates in respiratory failure over a period ranging from several years to a decade or more (12). This implies a requirement for continuous maintenance replacement therapy beginning early in the course of the disease. In contrast, the use of factor VIII preparations in hemophilia and C1-inhibitor preparations in hereditary angioedema can be timed to provide control of readily observable expressions of disease, e.g., bleeding and mucosal edema, respectively. Whereas long-term maintenance replacement therapy is used for the control of recurrent infections in hypogammaglobulinemic patients, this has proven feasible because immunoglobulin G has a serum half-life of approximately 30 days in humans (9). In contrast, studies of the metabolism of alpha-1-antitrypsin in man have shown that it possesses a half-life of only 4 to 5 days (13). Taken together, these data suggest that successful parenteral replacement therapy of alpha-1-antitrypsin deficiency would require large amounts of alpha-1-antitrypsin given at frequent intervals to assure continuous protection of the alveolar structure in the deficient individual. In addition, safe and effective alpha-1-antitrypsin replacement would require a preparation of the antiprotease that would: (*1*) have access to the target organ, i.e., the lung; (*2*) retain its biologic activity; (*3*) be sterile and pyrogen-free; (*4*) be devoid of serum components likely to produce adverse reactions when administered intravenously (e.g., aggregates of IgG); and, (*5*) offer an acceptable margin of safety with regard to the risk of transfusion hepatitis (14).

As a necessary concession to the stringent requirements imposed by the biology of alpha-1-antitrypsin, we approached the problem of replacement therapy by partially purifying the protein for use in replacement therapy by sequential polyethylene glycol precipitation of pooled normal plasma (14). This approach permits the recovery of approximately 80% of the alpha-1-antitrypsin in the 20- to 40% polyethylene glycol fraction of the plasma. Rapid processing allows the preparation of an alpha-1-antitrypsin concentrate that is sterile and pyrogen-free while retaining its full antiprotease activity. The initial treatment of the pooled plasma with 20% polyethylene glycol results in the removal of aggregates of IgG, high molecular weight kininogen, and complement components C1, C4, C2, C3, and C5. In addition, this form of precipitation in polyethylene glycol confers a desirable margin of safety with regard to the risk of transfusion hepatitis, because hepatitis B virus is removed from the alpha-1-antitrypsin fraction under these conditions. The final alpha-1-antitrypsin concentrate represents a 3-fold enrichment of the antiprotease. Importantly, this enrichment was attained without resorting to large scale chromotographic procedures with the attendant risk of contamination with bacterial products. The 20- to 40% concentrate is sterilized by microfiltration and can be stored for up to two weeks prior to use in intravenous replacement therapy.

The goals of our initial studies of parenteral alpha-1-antitrypsin replacement have been directed toward objective evidence of reversal of the protease-antiprotease imbalance within the PiZ lung; i.e., if there is any promise in the specific therapy of this disease, it is logical to hypothesize that parenteral replacement therapy must reestablish protease-antiprotease balance at the level of the lower respiratory tract when administered at reasonable intervals.

In order to attain a level of the serum antiprotease similar to that of normal individuals, the patients with the PiZ phenotype would require nearly tenfold increase in

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their serum alpha-1-antitrypsin levels. However, analysis of the epidemiology of destructive lung disease associated with the inheritance of "intermediate" forms of alpha-1-antitrypsin levels has shown that levels in the range of 50% of normal (e.g., PiMZ phenotype) possess a minimal risk for the devleopment of disease (15). By contrast, individuals with the PiSZ phenotype have serum alpha-1-antitrypsin levels in the range of 30 to 35% of normal and clearly have an increased risk of destructive lung disease. Thus, it is logical to hypothesize that serum alpha-1-antitrypsin values in the range of 35- to 50% of normal is all that is necessary to maintain adequate protease-antiprotease balance at the level of the alveolar structures. In this context, we designed our replacement therapy based on the known extracellular distribution of alpha-1-antitrypsin and its catabolic rate (13) in order to maintain serum and lung levels in this theoretical protective range. To accomplish this, 4 g of alpha-1-antitrypsin were administered intravenously every 7 days for 4 consecutive weeks. Documentation of the biochemical effects of the replacement therapy was provided by analysis of bronchoalveolar lavage fluid obtained before therapy and again following the second and fourth infusion of the alpha-1-antitrypsin concentrate.

Results and Discussion

Five individuals with severe alpha-1-antitrypsin deficiency (PiZ phenotype; mean serum alpha-1-antitrypsin levels of 37 ± 4 mg/dl) and advanced destructive lung disease were enrolled in the replacement therapy study (14). The alpha-1-antitrypsin levels were increased from mean pretreatment values of 37 ± 4 mg/dl to 108 ± 12 mg/dl at 2 days following the intravenous infusion of 4 g of alpha-1-antitrypsin. There were commensurate increases in serum anti-elastase activity. Thus, each of the study patients demonstrated the anticipated increment in functional and antigenic serum alpha-1-antitrypsin levels following the weekly infusions.

Whereas serum alpha-1-antitrypsin levels fell progressively during the 7-day interval between infusions, the weekly infusion schedule maintained levels in a range at or above 35% of normal levels. This implies that serum levels of alpha-1-antitrypsin necessary to protect the alveolar structures can be maintained with this replacement schedule.

More importantly, the use of intravenous alpha-1-antitrypsin administration resulted in a restoration of antiprotease balance at the level of the lung in these PiZ subjects. Bronchoalveolar lavage analysis prior to therapy demonstrated an absence of functional antielastase activity, in keeping with alpha-1-antitrypsin levels that were less than 5 μg/mg albumin (normal values; 51 ± 10 μg/mg albumin) (7, 14). In contrast, two days following the intravenous administration of four g of alpha-1-antitrypsin, lower respiratory tract alpha-1-antitrypsin levels increased to approximately 60% of normal along with the appearance of an equivalent quantity of functional antielastase activity. In addition, whereas active elastase activity was recovered from the lower respiratory tract prior to the initiation of replacement therapy, the infusion of alpha-1-antitrypsin concentrate abolished the free elastase activity within the alveolar structures of these individuals (8). Thus, the use of bronchoalveolar lavage to monitor the effects of replacement therapy demonstrated that parenteral administration of alpha-1-antitrypsin restored protease-antiprotease balance at the level of the lung in PiZ patients. These findings suggest that it is feasible to interrupt the proteolytic processes operative in the pathogenesis of this lung disease by weekly administrations of an alpha-1-antitrypsin concentrate readily obtained from pooled human plasma (8, 14).

Experience with this alpha-1-antitrypsin preparation also demonstrated the safety of this approach to replacement therapy. There were no significant untoward reactions to the infusion of the alpha-1-antitrypsin concentrate. The use of carefully screened volunteer plasma coupled with the 20% polyethylene glycol precipitation appear to provide a desirable margin of safety with regard to the risk of transfusion hepatitis. None of the patients in this study developed clinical or biochemical evidence of hepatitis following the infusion of this plasma fraction (14).

The next step in the evaluation of replacement therapy in alpha-1-antitrypsin deficient emphysema is the application of this approach to long-term clinical studies. Before replacement therapy can be accepted for routine use in this disorder, studies are necessary to demonstrate that the PiZ phenotype will result in conservation of lung function and an improved clinical outcome in alpha-1-antitrypsin deficient individuals. Although such a study will be fraught with difficulty regarding selection of patients and clinical end-points, it will also provide a unique opportunity to test directly the relevance of the proteolytic theory of the pathogenesis of emphysema.

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Can Alpha-1-Protease Inhibitor Be Used in Replacement Therapy?[1-3]

CHARLES GLASER

SUMMARY We propose that alpha-1-protease inhibitor (α_1PI) can assume a major and beneficial role in preventing emphysema in α_1PI-deficient individuals, and may also prove of value in the treatment of adult respiratory distress syndrome (ARDS). α_1PI has a single, unusual disulfide bond that consists of a cysteine residue in the peptide chain covalently bound to a free amino acid cysteine. The linkage can be broken by reductants without adversely affecting the stability or the inhibitory activities of the protein. As a result of this property, α_1PI can be effectively separated in solution from many other plasma proteins by salting out the contaminants in the presence of strong reductants. We have applied the technique of reductive-salting out, coupled with more conventional DEAE-anion exchange chromatography to isolate α_1PI from Cohn Fraction IV-1, a relatively unused side product in the worldwide production of albumin and immunoglobulins. Furthermore, we have demonstrated that the product can be effectively pasteurized in the presence of various stabilizing additives. The necessary ingredients now exist for extensive clinical studies in the years ahead.

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Introduction

In this report, I will discuss the biology and chemistry of alpha-1-protease inhibitor (α_1PI, also called alpha-1-antitrypsin) in order to express the variables that must be considered to permit its use as a therapeutic agent.

First, the genetics of α_1PI will be presented as related to the problem at hand. In the discussion of its biology, the distribution, production, and clearance of α_1PI will be reviewed. Clinical states with apparent deficiencies of α_1PI will be listed, and our new method for isolation of α_1PI, which is compatible with the requirements of industrial-level plasma fractionation, will be discussed.

Finally, the pitfalls of employing such therapy will be described and alternatives will be briefly alluded to.

Genetic and Biologic Considerations

Proteolytic enzyme inhibitors are of fundamental importance in the regulation of key physiologic processes, including blood clotting and the dissolution of clots, blood pressure, hormone action, tissue remodeling and wound healing, the immune system, and various cellular processes including differentiation, division, recognition, and transformation (1-6). In plasma these inhibitors comprise about 10% of the total protein and include α_1PI (also called alpha-1-antitrypsin), α_2-macroglobulin, antithrombin III, α_1-antichymotrypsin, α_2-plasmin inhibitor, C_1-inactivator, and inter-alpha trypsin inhibitor.

α_1PI is the most prevalent of the protease plasma inhibitors. It is a glycoprotein of molecular weight ~ 50,000, and is also present in interstitial fluids, lymph, genital secretions, tears, saliva, nasal and pulmonary secretions, cerebrospinal and amniotic fluids, colostrum, platelets, and megakaryocytes. α_1PI is synthesized in liver parenchymal cells (7). It is secreted into the circulation in multiple forms as visualized by discontinuous starch gel electrophoresis (8) or isoelectric focusing (9), where as many as 8 discrete bands are seen. The gene locus is named Pi (for protease inhibitor) and the more than 20 genetic variants are given letter designations Pi B, C, D. . . M. . . Z, etc., according to their electrophoretic mobility. These alleles are expressed in an autosomal codominant fashion. The PiM allele is most common with a gene frequency of ~0.9, and individuals homozygous for this allele have ~1.3 mg α_1PI/ml of plasma (10).

Several of the variant forms of α_1PI are associated with decreased levels of circulating inhibitor. These include the alleles PiZ and PiS, as well as the less common Pi (null gene) (11), which is associated with little or no detectable product.

The pathophysiologic significance of low circulating levels of α_1PI derives from the strong association of pulmonary emphysema with the homozygous Z allele (12), and this can result from improperly regulated proteases released from inflammatory cells. Studies on the catabolism of PiM and PiZ forms of α_1PI indicate that both proteins have similar half-lives, about 5 days for the Z-protein (13), and 6 to 7 days for the M-protein (13, 14). Clearance from plasma for both forms of α_1PI is dramatically enhanced upon the loss of terminal sialic acid residues (15, 16), and this is apparently the result of affinity by the newly exposed galactosyl residues for a liver membrane receptor (15). Detailed investigation on a whole series of plasma glycoproteins indicates that the hepatic recognition and binding of sialic acid deficient molecules is a signal for transport to liver lysosomes where proteolytic breakdown occurs (17).

Feasibility of Replacement of α_1PI Deficient Subjects

As shown by Gadek *et al*, individuals who are homozygous for the deficiency gene Z have no effective antiprotease shield in their lower respiratory tract (18). α_2-macroglobulin, a large serum antielastase, and the bronchial mucus inhibitor, an antielastase of the central airways, do not protect the alveolar structure. In a follow-up study, α_1PI of ~ 17.5% purity was obtained from normal donors (Pi type MM) by fractional precipitations with polyethylene glycol (19). Five emphysematous individuals with severe α_1PI deficiency (Pi type ZZ) received weekly injections of 4 grams of α_1PI intravenously for 4 weeks. The treatment resulted in an elevation of serum α_1PI levels to ≥ 35% of normal (i.e., > 70 mg/dl at all times), and analysis of bronchoalveolar lavage fluid indicated that an effective antielastase activity was established within the alveolar structures. No problems were encountered with sterility, pyrogenicity, or hepatitis. Also, none of the patients showed any antibody formation against PiM protein up to one year following infusion. Finally, no signs of allergic reaction or volume overload were found. These results are very encouraging, although the introduction of α_1PI at the low level of purity used by the authors is impractical for long-term treatment, because the infusion of such a protein load (22.9 g/285ml/week) required 6 to 8 hours.

Would α_1PI be a Useful Therapeutic Agent in Other Diseases?

It has been demonstrated that unrestrained proteolysis occurs in the lungs in patients with adult respiratory distress syndrome (See Cochrane *et al*, this symposium). In addition, active neutrophil proteases have been demonstrated in purulent secretions of patients with chronic bronchitis (20), in patients with pneumonia (see Cochrane *et al* and Lonky, this symposium) and free proteases have been suggested as having a pathogenetic role in pancreatitis. In addition, investigators have not determined whether the liver diseases seen in infants with PiZ phenotype might be favorably af-

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[2] Supported by NIH Grants HL-23357, HL-24861, and HL-21271 from the National Heart, Lung, and Blood Institute.

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TABLE 1

PURIFICATION OF α_1-PROTEASE INHIBITOR FROM COHN IV-1 BY CONVENTIONAL MEANS*

	Protein (mg)†	α_1-PI (mg)‡	Specific TIC (mg/mg)†§	Activity Recovered (%)‖	Purification Factor
Cohn fraction IV-1	14,000	1,490	0.059	100	1.0
DEAE-cellulose	2,240	1,020	0.18	68.5	3.1
QAE-sephadex	621	690	0.65	46.3	11.0
Concanavalin A	526	650	0.70	43.6	11.9
G-150 Sephadex	401	452	0.86 (0.85)¶	30.2	14.6

* Taken from reference 23.
† Determined by the method of Lowry, *et al*, (52).
‡ Determined by radial immunodiffusion and expressed as total mgs of antigen present in the fraction.
§ Trypsin inhibitory capacity divided by the protein concentration of the fraction in mg/ml.
‖ Based on the radial immunodiffusion assay.
¶ Based on $E^{0.1\%}_{1\ cm,\ 280\ nm} = 0.50$.

fected by raising plasma α_1PI levels to normal. Therefore α_1PI may have potential as a therapeutic agent for diseases other than emphysema.

Isolation of α_1PI on an Industrial Scale

α_1PI has been isolated in many laboratories as a relatively homogenous, stable, and active product suitable for research purposes (21–25). In deriving a product suitable for large-scale clinical application, however, many additional considerations become important including the source of starting material, the safety of each separation step employed, the feasibility of shifting each technique to large-scale processing, the reproducibility, and the cost. In addition, the product derived should be biologically potent, sterile, and safe (i.e., free of toxins, pyrogens, immunogens, carcinogens, teratogens, materials affecting blood pressure, and hepatitis). Beginning in 1974, we undertook the task of trying to overcome the various obstacles in providing sufficient α_1PI to the medical community for its clinical needs. The following is a report of our progress to date.

The most common large-scale plasma fractionation scheme in use today throughout the world continues to be the cold ethanol fractionation procedure of Cohn *et al* (i.e., Method 6) (26), or modifications thereof (27). In 1974, we received from Cutter Laboratories (Berkeley, CA) several Cohn Fractions for evaluation including II + III, IV-1, and IV-4. Each was provided as a frozen, homogenized paste. A preliminary evaluation revealed that IV-1 was most suitable for our purposes. This relatively unused byproduct is recovered during albumin and immunoglobulin production. It is formed as a precipitate in the fractionation process at ~ pH 5.2 and ~ 20% ethanol, and typically contains at least the following proteins: α_1PI, albumin, antichymotrypsin, antithrombin III, α-acid glycoprotein, ceruloplasmin, transferrin, α_1-lipoprotein, haptoglobin, inter-α-trypsin inhibitor, C_1-inactivator, α_2-macroglobulin, IgG, and IgA.

Approximately 10% of the protein present in the Cohn IV-1 paste is α_1PI as assayed by radialimmunodiffusion, and this corresponds to ~ 30% of the α_1PI in plasma. The distribution of α_1PI in other Cohn Fractions has not been described.

Our original purification scheme (23) involved activation of the inhibitor in slightly basic media, and successive column chromatographic steps on DEAE-cellulose, QAE Sephadex, Sepharose-bound Concanavalin A, and Sephadex G-150, and demonstrated the feasibility of isolating α_1PI by a method that could be directly applied to worldwide plasma fractionation schemes (table 1). It indicated in particular that most of the α_1PI that had been treated at pH 5.2 was not permanently inactivated, although later work (unpublished) in our laboratory shows that, for some lots of Cohn IV-I, as much as 30% of the α_1PI is in an aggregate form that is irreversibly inactivated.

The finding that α_1PI could be recovered in active form from low pH solutions was in contrast to the reports of others who claimed that low pH conditions would permanently inactivate α_1PI. To investigate this matter in more detail, we initiated a study of the low pH inactivation of α_1PI (28). Our investigation indicated that active α_1PI could be stored at pH 2–4 at 4° C for variable time periods with eventual recovery of considerable activity against trypsin and elastase. The inactivation by acid apparently occurs by two means:

(*1*) A rapid and largely reversible conformational change in the monomeric form of α_1PI that is accompanied by nearly complete loss of enzyme inhibitory properties and a large increase in the tryptophan fluorescence emission at 335 nm (figure 1). Reincubation of protein in basic media results in substantial recovery of activity within 1 h and this is preceded by a time-dependent decrease in the fluorescence measured at 335 nm. Almost all of the regenerated biological activity resides in the monomeric fraction that regains 91 to 98% of its original trypsin inhibitory capacity.

(*2*) The slow formation of higher molecular weight aggregates of α_1PI that have little or no enzyme inhibitory properties, and are not readily dissociated (table 2).

Although the isolation of α_1PI from Cohn IV-1 indicated promise, in practical terms it did not meet the necessary criteria for producing a useful blood product. The use of Sepharose-bound Concanavalin A is unacceptable, as the leakage of even minute amounts of Con A into the media could introduce the serious risk of a major antigenic reaction in the recipient. In addition, although the gel filtration step is safe and reproducible, it is not an approach that can be used for purification of greater than several grams at a time, and therefore is not practical.

Concurrently, we investigated the chem-

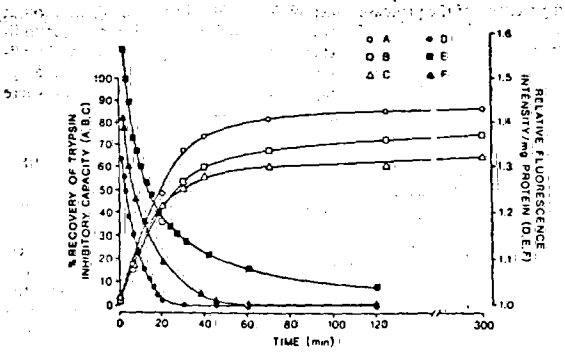

Fig. 1. The increase in trypsin inhibitory capacity (A, B, C) and corresponding decrease in the fluorescence intensity (D, E, F) as a function of time following titration of various acidic solutions to pH 8.0–8.2 with an equal volume of 0.66 M Tris buffer, pH 8.5. The original solutions were as follows: A, D (0.1 M glycine/HCl, pH 3); C, F(0.1 M glycine/HCl, pH 2); B, E(0.1 M ammonium acetate, pH 4). The acid incubation period was 30 min. The recorded fluorescence intensity in each case is relative to that of a control solution prepared by dissolving α_1PI in a pH 8.0–8.2 buffered solution consisting of one part of the appropriate low pH solution and one part of 0.66 M Tris HCl, pH 8.5. Both the trypsin inhibitory capacity (defined as 100%) and the relative fluorescence intensity (defined as 1.0) remained constant during the time course of the experiment when measured using a control α_1PI.

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TABLE 2

PERCENT TRYPSIN INHIBITORY CAPACITY RECOVERED FROM SEPHADEX G-150 COLUMNS AFTER LOW pH INCUBATION*

	Monomer		Polymer	
	30 min	48 h	30 min	48 h
0.1 M glycine/HCl buffer, pH 2.0	98† (63)‡	72 (46)	< 2	< 10
0.1 M glycine/HCl buffer, pH 3.0	93 (75)	94 (58)	< 2	< 10
0.1 M ammonium acetate, pH 4.0	91 (78)	84 (36)	< 2	< 10

* Taken from reference 28; following incubation at low pH for 30 min or 48 h, solutions were dialyzed to pH 8.0 for 24 h.
† % of trypsin inhibitory capacity recovered relative to control solution of α_1PI that has not undergone acid treatment.
‡ Figures in parentheses represent the % of α_1PI recovered in the monomer fractions based on $E^{0.1\%}_{1\text{ cm},280\text{ nm}} = 0.50$.

istry of the disulfide bond of α_1PI (29). α_1PI has a single, unusual disulfide bond that consists of a cysteine containing region (i.e., Phe-Asn-Ile-Gln-His-Cys-Lys) within the polypeptide chain which is covalently linked to free cysteine or glutathione via a disulfide bridge. When this disulfide bridge was broken by reduction and then alkylated with iodoacetamide, the resultant product was found to be fully functional as an inhibitor of trypsin and elastase in esterolytic and proteolytic assays. The modified protein was also found to be identical to native α_1PI when analyzed by immunologic, electrophoretic, and spectral methods.

These findings concerning the disulfide bond of α_1PI prompted us to explore a new approach to the purification. We treated a mixture of plasma proteins containing α_1PI with a strong reductant (e.g., dithiothreitol, DTT). The disulfide bridge in α_1PI should be reduced along with large numbers of disulfide bonds in many of the other proteins in the media. Because it is well known that S-S bridges help to stabilize proteins in solution, many if not most of the undesired proteins should be destabilized by this reductive treatment and made more amenable to precipitation [e.g., in $(NH_4)_2SO_4$ or polyethylene glycol]. We had earlier determined that we could effectively remove ~ 70% of the contaminating protein with a recovery of 70 to 75% of α_1PI by salting out the reactivated Cohn IV-1 mixture in 50% $(NH_4)_2SO_4$. We used this condition to test the effect of treatment of the Cohn IV-1 media with various concentrations of DTT at 25° C for 24 h before salting out at 4° C with $(NH_4)_2SO_4$, stirring overnight and centrifuging. In addition, in some experiments, we preincubated the media with 2.5% by weight of Aerosil 380 (Degussa, Inc., Teterboro, NJ), a fumed silica product that selectively adsorbs several plasma proteins including α- and β-lipoproteins and plasminogen (30, 31).

The reductive salting out technique is highly effective at concentrations of 0.025 M DTT or higher and the addition of Aerosil to the media gives an additional purification, as illustrated in figure 2. Centrifugation of the Aerosil-DTT-$(NH_4)_2SO_4$-treated Cohn IV-1 solution effectively removes ~ 95% of the overall protein from the media (figure 2B), giving a purification factor of nearly 14 compared with ~ 8 for product obtained after treatment with DTT and $(NH_4)_2SO_4$ but no Aerosil, and only 3 for product obtained by conventional salting out (~ 50% $(NH_4)_2SO_4$) (figure 2A). Albumin, which is present in ~ 15% in the Cohn IV-1 media, is completely removed by the reductive treatment with or without Aerosil (figure 2C). Approximately 70 to 75% of the α_1PI was recovered from the treatment in all cases (figure 2D). Similar matrices were conducted to optimize pH, temperature, and incubation time of the reducing step (data not shown). Neither cysteine nor mercaptoethanol at concentrations as high as 1.0 M could approximate the results found with DTT, indicating the

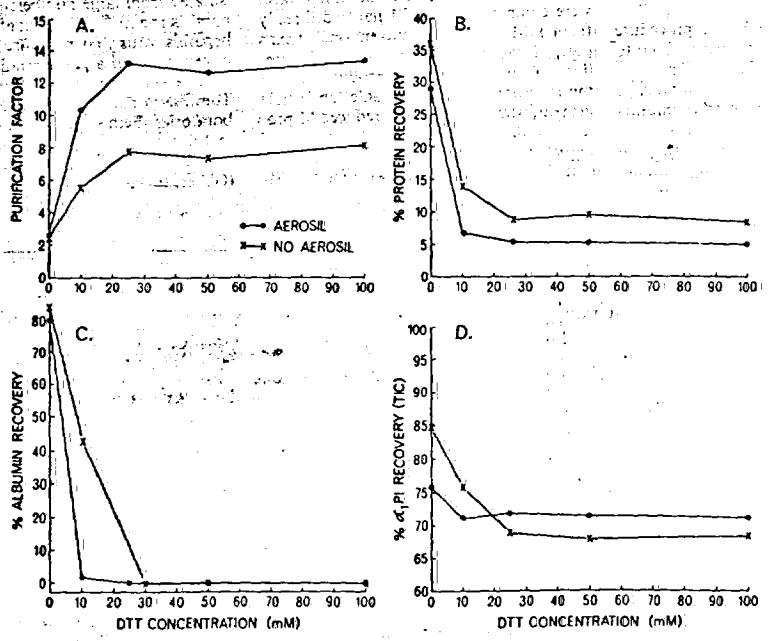

Fig. 2. Purification profile of α_1PI from Cohn IV-1 as a function of dithiothreitol concentration. A. Purification factor; B. Percent total protein recovery; C. Percent albumin recovery; D. Percent α_1PI recovery measured by TIC.

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special importance of this reducing agent that results from its ability to form a stable 5-membered ring on oxidation, shifting the equilibrium toward a reduced product.

When we had determined the proper conditions, Cohn IV-1 paste was activated, treated with Aerosil and DTT, and salted out with $(NH_4)_2SO_4$ according to the following scheme (32):

50 g of Cohn IV-1 (15 g protein, 1 g active α_1PI) dissolved in 0.5 L of 0.1 M Tris-HCl, pH 8.5, 24 h, 4° C.

2.5% dry Aerosil (final concentration), followed by 0.025 M DTT, stir for 24 h at 25° C.

50% saturated $(NH_4)_2SO_4$, final concentration, stir 16 h at 4° C.

Spin (5,000 × *g*, 30 min), wash, spin, concentrate to 300 ml by ultrafiltration (10,000 M.W. cut off).

Dialyze against 4L 0.01 M cysteine, 0.01 M phosphate (pH 7.6) at 25° C, then phosphate buffer alone.

The dialysate was then loaded onto a DEAE-cellulose column equilibrated with 10mM sodium phosphate (pH 7.6) to a concentration of 40–75 mg protein/ml of resin. The column was washed with equilibrating buffer until the $OD_{280\,nm}$ approached zero, and then α_1PI was eluted with a gradient of 10 mM phosphate (pH 7.6) to 10 mM phosphate (pH 6.0), 0.1 M NaCl. Fractions having a specific activity of > 0.90 mg/mg were pooled, dialyzed against 10 mM NH_4HCO_3, and lyophilized (figure 3).

As indicated in table 3, the procedure yields a highly purified product with 40 to 45% recovery. Analysis of the final product using radial immunodiffusion against a large number of specific antibodies to plasma proteins reveals only antithrombin III as an impurity (< 1%). In several selected experiments, a specific elastase inhibiting activity of ~ 99% is found, compared with a standard α_1PI preparation (MM type).

Figure 4 shows an SDS-polyacrylamide gel electrophoresis pattern of Cohn IV-1 (4B) and of several partially purified fractions derived from salting-out (with and without Aerosil and/or DTT [4C, 4E, and 4F], and following DEAE-cellulose chromatography [4G]. The product obtained by Aerosil-DTT-$(NH_4)_2SO_4$ treatment (4E) compared with conventional salting out (no Aerosil, no DTT) (4C), or to salting out in the presence of Aerosil (4D), indicates the effectiveness of DTT in the isolation scheme. The ability of the Aerosil to remove most of the lower molecular weight impurities is seen in 4E (with Aerosil) versus 4F (without Aerosil), and is important as these bands are sometimes associated with blood-pressure regulating substances. Aerosil effectively removes all lipoprotein at the reductive-salting-out step and additionally improves the removal of ceruloplasmin and transferrin, as shown by radialimmunodiffusion. The final product (4G), obtained following the DEAE-chromatography, appears homogeneous and migrates with the same mobility as a control sample of M-protein purified from plasma by conventional means (4H).

To evaluate whether cysteine dialysis results in the reforming of the α_1PI disulfide bridge in the final product, the extent of incorporation of ^{14}C-containing iodoacetamide was measured for both reduced and nonreduced protein, and the results also were compared with α_1PI purified directly from PiM plasma by conventional means (i.e., without reductive-salting out). The reduced Cohn IV-1 derived product showed the incorporation of 0.80 mole label/mole protein, whereas that of the reduced M-protein was 0.92 mole/mole. In the absence of reductant, the Cohn product showed 0.06 mole/mole incorporation whereas the M-protein showed 0.02 mole/mole. These results indicate that the α_1PI derived by the reductive-salting out method has a protected thiol function (i.e., nonreactive in the absence of reductants), presumably in the form of disulfide bond with cysteine, since little or no dimer or aggregate is seen.

Phenotyping of the product gave a pattern indistinguishable from the Pi-type M product. This is not a surprising result for a pooled plasma pattern because of the high gene frequency of the M-protein.

The LD_{50} in mice and the blood pressure effects in anesthetized rats were used to compare α_1PI prepared in Cutter Laboratories by a procedure similar to our own to α_1PI purified by more conventional procedures. The LD_{50} indicated a low toxicity. In rats the more pure α_1PI preparations (both DTT-treated and conventional) were nonhypertensive and the functional elastase inhibitor capacity and antigenic half-lives of all preparations were similar (D. Schroeder, Cutter Laboratories, private communication).

The reductive-salting out is applicable to other fluids and this is illustrated in table 4 for diluted human plasma. A purification factor of ~ 11 was achieved in the presence of reductant whereas no purification was found in its absence (no Aerosil was used in these experiments).

For long-term treatment of α_1PI-deficient individuals, a major limitation to the use of this inhibitor, or any other plasma product isolated from large numbers of human donors, is the high risk of contamination by hepatitis virus. To minimize this risk, we have developed a pasteurization procedure in collaboration with Drs. Ken Ingham and Tom Busby at the American Red Cross Laboratories, Bethesda, MD. A series of thermal denaturation profiles of α_1PI was obtained using various stabilizing molecules (Glaser, Ingham, unpublished observations).

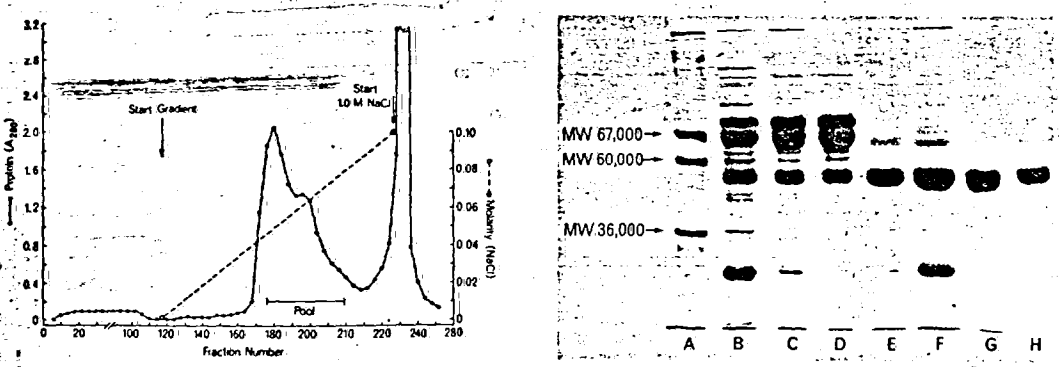

Fig. 3 (*left*). Chromatography of α_1PI obtained from purification step with Aerosil-DTT-$(NH_4)_2SO_4$ on DEAE-cellulose. The column (13 × 1.5 cm) was equilibrated with 0.01 M sodium phosphate (pH 7.6). After application of the sample, the column was washed with equilibration buffer and a linear gradient to 0.01 M sodium phosphate + 0.1 M NaCl, pH 6.0, was initiated at fraction 118 (500 ml total). Fig. 4 (*right*). Gel slab electrophoresis of samples from successive steps in the purification of α_1PI from Cohn IV-1. A. M.W. standards (Pharmacia); B. Cohn IV-1 (40 μg); C. Supernatant after 50% $(NH_4)_2SO_4$ precipitation (30 μg); D. Supernatant after 50% $(NH_4)_2SO_4$ and 2.5% Aerosil (30 μg); E. as in D, plus 25mM DTT (30 μg); F. 50% $(NH_4)_2SO_4$ and 25 mM DTT (30 μg); G. α_1PI from Cohn IV-1 after DEAE cellulose chromatography (20 μg); H. α_1PI (Pi type MM) isolated from human serum by conventional methods (20 μg).

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TABLE 3

PURIFICATION OF α_1-PROTEASE INHIBITOR BY THE REDUCTIVE-SALTING OUT METHOD*

	Protein mg	α_1PI Corr (TIC)	Specific Activity mg active α_1PI / mg total protein	α_1PI Yield %	Purification Factor
Cohn IV-I	16,050†	900	0.056	—	—
Aerosil + dithiothreitol + $(NH_4)_2SO_4$	770†	531	0.692	59	12.4
DEAE-cellulose	391‡	387	0.990	43	18.9

* Taken from reference 32.
† Determined by Lowry with a BSA standard.
‡ Determined by using $E^{0.1\%,\ 1\ cm}_{280\ nm} = 0.50$.

The increase in 1,8-anilinonaphthalene sulfonate (ANS) binding as a consequence of denaturation was used to monitor each experiment (33).

Solvolytic conditions that resulted in substantial shifts to higher temperatures in the ANS binding curves, especially where the onset of denaturation was above 60° C, were considered for further evaluation. Under selected solvolytic conditions, α_1PI was heated for 10 hours at 60° C and its activity was measured. Pasteurization in the presence of combinations of citrate (1.2M) and either EDTA (0.5 M) or gluconate (1.2 M) results in a product that is 90 to 95% active against trypsin and elastase. Aggregation accounts for some, but not all, of the activity loss. Loss of activity is not affected by protein concentration. Reductant or free radical scavengers in the media (mercaptoethanol, ascorbic acid, 2,6-di-tertbutyl-4-methylphenol [BHT]) do not increase the yield of active product.

In conclusion, our work has shown that α_1PI can be obtained as a blood product by means that are inexpensive, simple, safe, and reproducible, and which give a relatively pure product (> 90%) in high yield by procedures that are applicable to large scale industrial processing. Although we carried out the DEAE-cellulose separation via column chromatography, batch-mode processing should certainly be achievable although some loss of purity would be expected. In addition, we have developed a pasteurization protocol that results in the preservation of 90 to 95% of α_1PI activity following heat treatment at 60° C for 10 hours.

Potential Hazards of α_1PI Replacement Therapy

Because the consequences of a severe deficiency of α_1PI are pulmonary emphysema in ~ 80% of the affected individuals, then a strong logic exists for prevention of this disorder with α_1PI replacement therapy. In this regard, it is appropriate to compare the structure of the M protein to its Z variant.

An amino acid substitution of glutamic acid in the normal protein to lysine in the variant protein was found by peptide fingerprinting (34, 35). A second substitution of glutamic acid to glutamine also was reported by one of the groups (35). In addition, the Z protein may contain less carbohydrate than the M protein. In one study, a possible sugar difference was found for residues of sialic acid, the terminal sugar in most plasma glycoproteins (36), and in other reports the Z protein also was found to be deficient in glucosamine and the neutral sugars mannose and galactose, as well as sialic acid (37, 38). In experiments from this laboratory involving successive treatment with neuraminidase and galactose oxidase, followed by reduction with tritium-labeled sodium borohydride and isolation of the neutral sugar fraction, it was shown that the Z protein has a diminished content of both galactose and sialic acid relative to the M form, and additional experiments indicate that neither protein contains terminal galactosyl residues (15). The question of carbohydrate differences is still unresolved, however, because a recent investigation revealed no carbohydrate differences between the two forms (39).

The two proteins were compared by circular dichroism spectroscopy and by fluorescence quenching experiments using anionic (I^-) and neutral (i.e., acrylamide) probes (40). Both proteins share a similar secondary structure, i.e., ~ 45 to 50% α-helix and 15 to 20% β-structure. Evidence was accumulated to show that the microenvironment in the vicinity of the 3 tryptophanyl residues is altered in Z form compared with the M form as shown by (*a*) the absence of the positive dichroic band in the 290 to 300 nm region of the circular dichroism spectra, (*b*) a > 50 % increase in quantum yield in the tryptophanyl fluorescence emission spectra, (*c*) an increased accessibility of tryptophan to quenching by iodide, and (*d*) acrylamide quenching experiments which indicate that all tryptophanyl residues in the Z protein are quenched equally or that quenching is dominated by a single residue, whereas in the M protein, heterogeneous quenching occurs.

As discussed by Boyer *et al* (41), individuals with mutant genes that result in the absence of production of a particular protein are especially likely to be immunologically intolerant of such a protein. Thus, in terms of protein replacement, the presence of small levels of the protein native to the recipient is distinctly better than none. We can distinguish between cross-reactive negative (CRM−) mutants (i.e., those individuals with no recognizable version of a particular protein) and cross-reactive positive (CRM+) mutants at the same locus. Because most CRM+ mutants (PiM vs. PiZ) involve only one amino acid residue substitution out of hundreds, the risk of immunologic recognition of the altered sequence or of any consequent local conformational changes imposed by the sequence variation should, in general, be much less severe than in CRM− individuals. In CRM− individuals, all peptide regions and conformational arrays are potential antigenic sites. Also, it should be pointed out that some CRM− mutations may turn out to be CRM+ using more sensitive means of detection. Just such a finding appears likely for at least some cases attributed to the Pi-null gene, where an electrophoretic pattern identical to that of very dilute PiM serum could be produced by radioautography (42). Thus far, limited experience with the repeated infusion of M protein into Z recipients gives no indication that the M protein contains any novel antigenic sites compared with the Z-variant. It should be noted that a possible antigenic reaction is a legitimate concern for long-term replacement therapy in α_1PI-deficient individuals, but it is not an important issue for potential treatment of ARDS.

Of course, many questions remain concerning the use of α_1PI in replacement ther-

TABLE 4

THE PARTIAL PURIFICATION OF α_1-PROTEASE INHIBITOR FROM PLASMA BY REDUCTIVE-SALTING OUT*

0.1 M dithiothreitol	Dilution	Protein† Initial	Protein† Final	% Recovery (Protein)	% Recovery of α_1PI‡	Purification Factor
+	2-fold	501.0	46.2	9	61	6.66
+	4-fold	250.5	17.9	7	68	9.60
+	6-fold	167.0	10.6	6.3	72	11.40
−	2-fold	501.0	307.8	61.0	65	1.07
−	4-fold	250.5	161.0	64.0	78	1.22
−	6-fold	167	121.6	73.0	81	1.12

* Taken from reference 32.
† Determined by Lowry.
‡ Determined by trypsin inhibitory capacity.

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apy. These include the conditions of storage, the concentration of protein, the degree of purity, the pH, and the infusion rate. One must carefully monitor blood pressure effects that could be introduced by minor contaminants from the kallikrein-kinin system (43, 44). One also must consider the possibility that any aggregates formed on storage or heating of the protein will introduce new antigenic determinants in the system as have been reported for albumin (43, 45) and immunoglobulin (44). In the studies of Gadek *et al* (18), the concentration of total protein employed was ~ 8.0 g/100 ml and the osmolarity of the preparation was 450 mosmol/l. If an individual required 4.0 g of α_1PI/ week and the preparation used was 80% pure and introduced at a comparable concentration, intravenous injection would need to be 60 to 65 ml. Other routes of delivery such as albumin beads (46) or aerosol need to be considered but would appear to be incapable of providing sufficient concentrations of the protein to the lower tract of the lung.

Practical Considerations and Possible Alternatives

It is likely that replacement therapy with α_1PI would stop the progression of lung disease in nonsmoking individuals with α_1PI deficiency. Such a conclusion was reached by a National Institutes of Health Working Group for the evaluation of elastase inhibitor replacement therapy in pulmonary emphysema held in 1978 in Bethesda, MD (47). The PiM-allele is by far the most common, followed by PiS and PiZ (48). At the present time, most of the severely deficient individuals in the U.S. have not been identified. Mass screening can be achieved, however, and in Sweden all newborn infants are tested routinely (49). In order to assay large populations, a preliminary measurement of α_1PI levels by radial-immunodiffusion should provide an effective cutoff point. Individuals with perhaps 40% or less of the normal levels could then be phenotyped.

Early detection of a deficiency state could provide a powerful motivation to individuals to avoid lung irritants including smoking, working in industrial jobs where there are irritating fumes, dust, smoke, and proteolytic aerosols (e.g., enzyme soaps), and to seek early treatment for respiratory tract infections. Long-term α_1PI therapy could be commenced in deficient individuals during early stages of lung dysfunction. This might be detected by pulmonary function tests including spirometry and the pulmonary diffusing capacity for carbon monoxide (single breath (DL_{CO})) or by chemical tests such as the radioimmunoassay of elastin degradation products, as described in this symposium by Dr. Umberto Kucich.

Estimates of the number of individuals seriously deficient (i.e., < 40% of normal levels) in α_1PI vary. A conservative estimate of 1 in 2,000 to 3,000 individuals in the United States would indicate that perhaps 100,000 to 150,000 individuals are eventual candidates for α_1PI therapy. If perhaps 50% of these individuals could be identified, and if early signs of lung dysfunction are the criteria for replacement therapy, perhaps 25,000 individuals would be potential recipients. In the United States, approximately 3 million liters of plasma yielding 4 million grams of α_1PI are collected yearly (50). With eventual recovery of 40% of this protein from Cohn IV-1 and other plasma fractions, 1.6 million g/year or 32,000 g/ week would be available. Because ~ 4 g/ week are needed per ZZ homozygote to maintain adequate inhibitor levels at all times, a significant fraction of the needs of the community could be met if all α_1PI were used for this purpose.

Of course, the presentation of Dr. Cochrane in the symposium points out the exciting possibility that α_1PI might prove beneficial in controlling ARDS and, if this proves correct, significant supplies of α_1PI would be needed for this purpose. At some point it may become feasible to enhance the supplies of α_1PI through recombinant DNA techniques, and considerable progress has been made in the cloning of α_1PI (51). The large quantities of protein needed/patient as compared with other current projects under way in that industry, however (e.g., growth hormone, insulin, interferon), would suggest an unfavorable cost factor compared with the facile isolation of α_1PI from plasma fractions. In addition, and perhaps even more important, α_1PI is a glycoprotein containing ~ 13% carbohydrate in 3 complex carbohydrate chains. α_1PI produced by recombinant techniques using bacteria or yeast will be either devoid of carbohydrate, or would have modified sugar side chains compared with the normal circulatory inhibitor, and such α_1PI derivatives would need to be evaluated extensively with regard to solubility, activity, clearance rates, antigenicity, etc., prior to clinical use. If these problems can be overcome, however, the reductive-salting out approach to purification should be applicable to an α_1PI product obtained by genetic engineering technology.

Although emphysema in smokers and others exposed to environmental hazards also appears to result from an overwhelming of the antiprotease shield in the lung, the limits of availability of α_1PI probably would place these individuals in a very low priority to receive the drug since other more effective means of intervention (e.g., cessation of smoking) are possible. The eventual introduction of low M.W. synthetic elastase inhibitors might also aid these individuals provided highly specific drugs can be developed that are effective, nontoxic, and long-lasting. This subject is addressed by Dr. James Powers in this symposium.

In conclusion, scientific studies from many laboratories suggest the likelihood that α_1PI can assume a major and beneficial role in preventing emphysema in α_1PI-deficient individuals and may also prove of value in the treatment of ARDS. The industrial production of an active and safe α_1PI product is now achievable, and extensive clinical trials are warranted in the years ahead.

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Synthetic Elastase Inhibitors: Prospects for Use in the Treatment of Emphysema[1-3]

JAMES C. POWERS

SUMMARY Human polymorphonuclear leukocyte elastase is the enzyme primarily responsible for the destruction of lung tissue observed in pulmonary emphysema. A number of potent reversible and irreversible inhibitors have been developed for human leukocyte elastase. Several of these inhibitors have been shown to be effective at preventing emphysema in animal models of the disease. There are excellent prospects for the development of a synthetic elastase inhibitor for use in treatment of human disease.

AM REV RESPIR DIS 1983; 127:S54-S58

Introduction

Human leukocyte (HL) elastase is suspected by many investigators to be the primary destructive agent in pulmonary emphysema. Other tissue proteases such as cathepsin G (1), the serine proteases from mast cells (2), and macrophage metallo-elastase may contribute to the damage. Indeed, cathepsin G appears to act synergistically with HL elastase and aids slightly in the solubilization of elastin ([3] and unpublished observations). However, these other enzymes must be given minor roles at best because their natural serum inhibitors (α_1-antichymotrypsin for cathepsin G and the mast cell chymotrypsin-like enzymes, and α_2-macroglobulin for the macrophage elastase) appear to be unaffected in individuals with emphysema.

α_1-Protease Inhibitor

The reactive site of α_1-PI has an Ala-Ile-Pro-Met*Ser-Ile-Pro-Pro sequence (4). The mechanism by which α_1-PI inhibits elastase and other serine proteases is not yet known, but it certainly involves interaction of the reactive site amino acids of α_1-PI with the active site of the protease. It is possible that the active site serine of elastase reacts covalently with the Met-Ser bond of α_1-PI to form a tetrahedral complex, an acyl enzyme, or simply interacts with no covalent bond formation, but this has not been definitely established. When complexes of proteases and α_1-PI are dissociated at high pH or by using nucleophiles (5), the Met-Ser bond indicated by an asterisk in the above sequence is cleaved.

We have synthesized a number of linear peptides with the amino acid sequence at the α_1-PI reactive site and shown them to be perfectly adequate substrates for HL elastase (6). The octapeptide Ac-Ala-Ile-Pro-Met-Ser-Ile-Pro-Pro-NH_2 is not an inhibitor of elastase but is an excellent substrate and is hydrolyzed at the Met-Ser bond with a K_M = 1.6 mM and k_{cat}/K_M = 10,000 $M^{-1}s^{-1}$. Oxidation of the Met in the octapeptide to methionine sulfoxide in Ac-Ala-Ile-Pro-Met(O)-Ser-Ile-Pro-Pro-NH_2 resulted in a twofold increase in K_M(K_M = 3.6 mM) and a 370-fold decrease in k_{cat}/K_M = 27 $M^{-1}s^{-1}$ at pH 7.5. Similar results have been obtained with other Met-containing peptides (7).

Therapy

Several approaches to treatment of emphysema can be envisioned. These include (*1*) use of α_1-PI, (*2*) use of other natural high molecular weight protease inhibitors from either plant or animal sources, (*3*) use of antioxidants to protect the α_1-PI of smokers (the role of oxidative processes in inactivating α_1-PI is discussed elsewhere in this symposium), and (*4*) use of low molecular weight elastase inhibitors. The use of α_1-PI in replacement therapy for PiZ patients has been reported (8) and is discussed in other reviews in this symposium. A number of other protease inhibitors that have been shown to inhibit HL elastase (9) also are candidates for this type of therapy. The disadvantage of these methods is the lack of oral activity of the protein protease inhibitors that are unlikely to survive and be absorbed from the digestive system. In addition, it probably will be difficult to obtain sufficient supplies of α_1-PI to treat the required number of patients with emphysema. The use of antioxidants to protect smokers appears to be a viable option, but has not been widely studied.

Low molecular weight elastase inhibitors have a number of advantages: (*1*) they could be obtained in sufficient quantities either by chemical synthesis or fermentation, (*2*) synthetic inhibitors could be rationally designed, (*3*) they would be non-antigenic, (*4*) they could be used orally or in aerosols, and (*5*) they would be readily absorbed and transported to the site of action. On the other hand, each new structure would require extensive toxicity testing in animals before approval could be obtained from the FDA.

Synthetic elastase inhibitors can be designed to bind either reversibly or irreversibly to elastase. Irreversible inhibitors have the advantage that elastase usually cannot become reactivated once the inhibition reaction takes place. Reversible inhibitors in contrast always must be present in sufficient concentration, and as the inhibitor is degraded or excreted, the elastase would regain its activity. A disadvantage of irreversible inhibitors is their possible increased toxicity due to reaction with other proteins in the physiologic medium. Irreversible inhibitors often contain reactive functional groups that can react with many side-chain functional groups of proteins.

It is conceivable that an elastase inhibitor could be targeted only for extracellular enzyme. This could be done by making alterations in the inhibitor structure that prevent or slow membrane transport. Such an inhibitor would be ideal for treating emphysema because HL elastase is only destructive outside of leukocytes. A targeted elastase inhibitor would prevent the deleterious effects of elastase although not altering its normal physiologic function.

Human Leukocyte Elastase

The elastase found in the granule fraction of human polymorphonuclear leukocytes is a typical serine protease belonging to the same family as more widely studied enzymes such as trypsin, chymotrypsin, and porcine pancreatic elastase. At present only small parts of the HL elastase sequence have been determined (10) and no X-ray crystallographic studies have been undertaken. Thus classification of HL elastase as a serine protease has been extremely valuable in terms of rational inhibitor design. Within a family of proteases, the catalytic residues in the active site of each member of the family are likely to be identical or very similar in structure and geometry. X-ray crystallographic studies on several serine proteases has shown this to be true even where the individual proteases such as chymotrypsin and subtilisin have evolved from different precursors. Thus it is possible to derive a fairly complete working model of the catalytic site of HL elastase from studies that have been carried out with better known members of the serine protease family such

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[2] Supported by grants from the National Institutes of Health (HL 18679) and the Council for Tobacco Research for basic aspects of the research.

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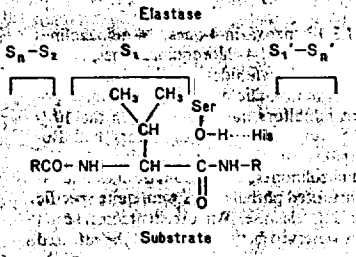


Fig. 1. A schematic drawing of the binding of a valine peptide substrate to the active site of HL elastase. The serine and histidine are 2 of the catalytic residues and the S_1 pocket is the primary substrate recognition site of the enzyme. S_n–S_2 and S_1'–S_n' are subsites that recognize other amino acid residues in an extended peptide substrate.

as bovine trypsin and chymotrypsin. This has allowed us and other investigators to direct inhibitors against specific functional groups in elastase's active site before sufficient enzyme or time was available to prove that those functional groups are present.

The substrate specificity of elastase is a poor predictor of the nature of its active site. Elastolytic enzymes are known that belong to at least 2 of the 4 protease families. These include serine proteases such as HL and porcine pancreatic elastase, and metalloproteases such as macrophage elastase and the elastase from *Pseudomonas aeruginosa*. In addition, enzymes such as thermolysin that are not usually considered elastases have high elastolytic activity. Indeed, classification on an elastase is not even informative in regard to the bonds cleaved in elastin because HL elastase and *P. aeruginosa* elastase have quite different substrate specificities.

A schematic representation of the active site of HL elastase is shown in figure 1. Based on crystallographic studies with other serine proteases (11), the catalytic residues are composed of a triad of amino acid residues, serine, histidine, and aspartic acid (not shown), that actually perform the peptide bond hydrolysis. In addition to the catalytic residues, the active site is composed of an extended substrate binding site that consists of a primary substrate binding site S_1 and various subsites on either side of the scissile bond. These subsites contribute to the binding of extended peptide substrates or inhibitors. The extended substrate binding site of HL has been shown to encompass at least 7 or 8 amino acid residues and has an equal number of subsites (6). The primary substrate binding site S_1 of HL elastase prefers to bind with substrates or inhibitors containing valine residues (12, 13). Porcine pancreatic elastase has a much smaller binding site and prefers alanine residues rather than valine.

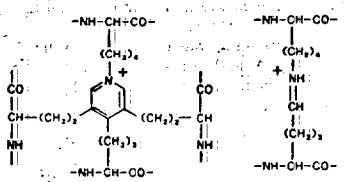
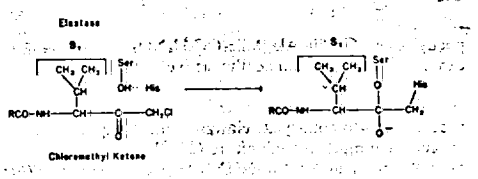


Fig. 2. Structure of 2 common elastin cross-linking residues, desmosine and dehydrolysinonorleucine. Isodesmosine is an isomer of desmosine where the substituents are attached to the 1, 2, 3, and 5 positions of the pyridinium ring.

Fig. 3. A schematic drawing of the reaction of a valine chloromethyl ketone with the active site of HL elastase. The enzyme is alkylated at the imidazole ring of the active site histidine residue. In addition, the serine OH adds to the carbonyl group of the inhibitor.



HL elastase effectively cleaves elastin, the flexible, highly cross-linked protein that is found in high quantities in mammalian lung and arteries. The chemical structure of elastin is not yet known, but fragmentary data available indicate that elastin has long sequences of small aliphatic amino acid residues such as Gly, Ala, Val and Pro. It also contains a number of cross-linking amino acid residues such as desmosine and isodesmosine (figure 2). These cross-linking residues are formed by the action of lysyl oxidase upon Lys residues in tropoelastin, a precursor of elastin. Studies with synthetic substrates containing model desmosine residues (14) support the hypothesis that HL elastase is a cross-link selective enzyme. In particular, HL elastase prefers to cleave sequences such as cross-linking amino acid residue $(AA)_n$-P_1*P_1' (asterisk indicates bond cleaved) where P_1 is Val or Ala and n is 1 or 2 but not zero. HL elastase also binds very tightly to elastin and the evidence indicates that this involves the cross-linked regions of elastin. In addition, it has been shown recently that HL elastase preabsorbed onto elastin is incompletely inhibited by α_1-protease inhibitor (15).

Human Leukocyte Elastase Inhibitors

Peptide chloromethyl ketones. The first class of selective inhibitors to be developed for HL elastase were peptide chloromethyl ketones (13, 16). These are active site-directed irreversible inhibitors for serine proteases. The inhibitors bind to the extended substrate binding site of the enzyme, and the reactive chloromethyl ketone functional group is then placed in the proper position to alkylate the active site histidine residue. In addition, the serine OH reacts with the inhibitor carbonyl group to form a hemiketal. A schematic drawing of the inhibition of HL elastase by a chloromethyl ketone is shown in figure 3.

The most effective chloromethyl ketone HL elastase inhibitor found thus far is MeO-Suc-Ala-Ala-Pro-ValCH_2Cl(k_{obs}/[I] = 1560 $M^{-1}s^{-1}$, MeO-Suc = CH_3OCOCH_2CO-) (13). This will not inhibit the other major leukocyte protease cathepsin G, but is an alkylating agent and will react slowly with nucleophiles such as glutathione (k_{obs}/[I] = 0.88 $M^{-1}s^{-1}$). This rate is quite slow and the inhibitor would discriminate in favor of HL elastase over glutathione by a factor of 1700 if the concentrations were equivalent.

The major problem with the use of peptide chloromethyl ketones *in vivo* is the potential for reaction at other sites. One possible approach to obtaining specificity involves the attachment of the inhibitor to a suitable carrier that could be targeted to the desired site of action. This has been accomplished with human albumin microspheres (HAM). HAM are nontoxic, nonantigenic and biodegradable, and because of their unique size are trapped in the pulmonary capillary bed after intravenous injection. An effective HL elastase inhibitor has been linked to HAM:

HAM-CONH-Spacer-Ala-Ala-Pro-ValCH_2Cl

The HAM-linked inhibitor was found to be capable of inhibiting elastase. When the inhibitor-HAM were injected into rats, they were rapidly and exclusively taken up by the lungs. Half of the modified HAM remained in the lung with a half-life of ca 17 days (17). Thus HAM offer a method for the delivery of elastase inhibitors to the lung.

Acylating agents. A number of HL elastase inhibitors have been developed that inactivate the enzyme by acylation of the active site serine residue. These include aza-peptides (18) such as Ac-Ala-Ala-Anle-OCH_2CF_3 (Anle = azanorleucine) that react with HL elastase to form stable acyl enzyme derivatives (figure 4). The acyl enzyme (a carbazate) formed from an azapeptide is much more stable to hydrolysis than the acyl enzyme (an ester) formed from a normal peptide substrate due to the electronic influence of the α-nitrogen atom in the azapeptide. In addition to acting as inhibitors, azapeptides such as the 4-nitro-

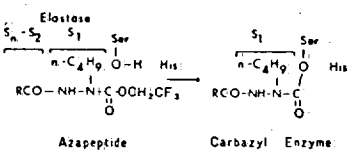


Fig. 4. Reaction of Ac-Ala-Ala-Anle-OCH_2CF_3 with HL elastase. The primary binding site (S_1) of the enzyme is shown interacting with the side chain of the azapeptide. The active site serine of elastase is acylated forming a carbazyl enzyme.

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phenyl ester Ac-Ala-Ala-Anle-$OC_6H_4NO_2$ can be used to determine the active site concentration of HL elastase (19). A group at Merck has also reported a series of azapeptides that do not acylate elastase but act instead as competitive inhibitors (20). For example, Ac-Ala-Ala-Pro-$NHN(CH_2)CO$-$OCH(CH_3)CO$-$NHCH_2C_6H_5$ has a K_I (dissociation constant of the enzyme inhibitor complex) of 2.2×10^{-5} M.

A group of heterocyclic acylating agents for HL elastase have been reported by Merck. These include N-acylsaccharins such as furyl saccharin ($ID_{50} = 3.6 \times 10^{-7}$M), N-acylbenzoisothiazolinones, and N-arylbenzoisothiazolinone, 1,1 dioxides (21, 22). These inhibitors acylate HL elastase by reaction between the active site serine residue of the enzyme and the heterocyclic ring carbonyl group. Some of the inhibitors show good selectivity toward HL elastase and others are fairly broad serine protease inhibitors.

A selective acylating agent for HL elastase could potentially be used therapeutically. A balance between the inherent reactivity of the acylating agent and potential side reactions such as hydrolysis or reaction with nucleophiles and its reactivity with HL elastase must be achieved. Some reagents such as alkyl isocyanates (23) are too reactive and nonselective for therapeutic use. Some azapeptides and acylsaccharins also appear to be unsuitable due to their hydrolytic instability. Thus the ideal inhibitor for further testing does not yet appear to be available.

Sulfonyl fluorides. Sulfonyl fluorides inhibit serine proteases by reacting with the active site serine residue to form a sulfonyl derivative (figure 5). We have discovered recently that introduction of fluoroacyl groups into a sulfonyl fluoride structure gives considerable reactivity and selectivity for HL elastase (24). HL elastase was inhibited most rapidly by 2-$(CF_3CF_2CONH)C_6H_4SO_2F$ ($k_{obs}/[I] = 1700$ $M^{-1}s^{-1}$) that is slightly better than the best peptide chloromethyl ketone for this enzyme. It also was quite selective and reacted with HL cathepsin G and bovine chymotrypsin at rates that were slower by 130- and 57-fold, respectively. One potential disadvantage of these 2-substituted sulfonyl fluorides is their instability toward hydrolysis. Whether this problem can be overcome and a useful agent produced still remains to be seen.

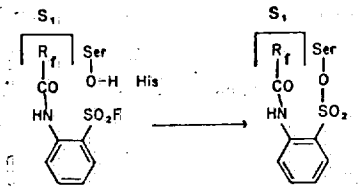

Fig. 5. Reaction of a 2-perfluoroacylamino benzene sulfonyl fluoride with the active site serine residue of HL elastase. R_f can be CF_3, CF_2CF_3, or $CF_2CF_2CF_3$.

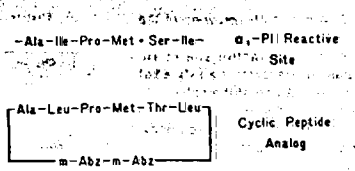

Fig. 6. Comparison of the reactive site sequence of α_1-PI with that of a cyclic peptide analogue. The bridging group is composed of 2 *m*-Abz (*m*-aminobenzoyl) moieties and is quite rigid.

α_1-protease inhibitor analogs. Crystallographic data have not yet been obtained on the plasma protease inhibitor α_1-PI, but X-ray structures have been obtained on several protein protease inhibitors from plant, microbial, and mammalian sources, and on 2 trypsin-trypsin inhibitor complexes. Only a small portion of the 2 inhibitors interact with the enzyme; most of the major contacts occur with the 6 or 7 amino acid residues in the immediate vicinity of the inhibitor reactive site. Comparison of the various protease inhibitor structures revealed a surprising degree of similarity in the conformation of the peptide chain at the various reactive sites even though the inhibitors inactivated proteases with quite different specificities. Therefore it is reasonable to assume that the conformation at the reactive site of α_1-PI bears some similarity to that of the other protease inhibitors. We have designed a number of inhibitors based on this premise.

A cyclic peptide analog of the α_1-PI reactive site is shown in figure 6 (18). This inhibitor was constructed by taking 6 amino acid residues in the α_1-PI reactive site and joining them with a bridging group that would allow those amino acid residues to occupy a conformation similar to that observed by X-ray crystallography in the reactive site of the soybean trypsin inhibitor. We substituted Leu for Ile for synthetic reasons because we decided it would not be a significant change. The Thr substitution for Ser was made at a time when α_1-PI was believed to contain either a Thr or Ser at that position.

The cyclic peptide inhibited HL elastase reversibly ($K_{II} = 0.39$ mM) and was quite specific because it did not inhibit porcine pancreatic elastase. A linear peptide Ac-Ala-Ile-Pro-Met-Ser-Ile-Pro-Pro-NH_2 with the α_1-PI reactive site sequence is a good substrate for HL elastase and has a K_M of 1.6 mM (6). The cyclic peptide binds more tightly to the enzyme than the linear peptide and was not cleaved by HL elastase even after long incubation times. The stability of the cyclic peptide analogs of α_1-PI toward HL elastase makes this class of compounds a potential useful group of inhibitors if sequence substitutions can be made that would lower the K_I value several fold.

Heterocyclic HL elastase inhibitors. HL elastase is inhibited competitively by heterocyclic compounds such as 2-substituted 4H-3,1-benzoxazin-4-ones, 4-quinazolines (figure 7), and 4-chloroquinazolines, N-substituted phthalimides and by thioesters of N-acylanthranilic acids (25). The most potent inhibitors have K_I values in the 10^{-7}-10^{-8} range. The best inhibitors had fluoroalkyl or fluoroacyl substituents. The quinazolinones, chloroquinazolines and N-substituted phthalimides were quite specific for HL elastase. An excellent correlation was observed between pK_I and the infrared carbonyl stretching frequency of the inhibitor. Thus the partially polarized carbonyl group of the inhibitor is interacting with the partially polarized catalytic triad (Ser-His-Asp) of HL elastase. The substituents on the inhibitor probably interact with the primary substrate binding site of elastase. This class of compounds eventually may be useful therapeutically.

Other reversible inhibitors. Quite a variety of other structures have been shown to be HL elastase inhibitors. These include the fermentation products elastatinal [$K_I = 5 \times 10^{-5}$ (26), $K_I = 8 \times 10^{-5}$M (12)] and elasnin [$ID_{50} = 3.3 \times 10^{-6}$M (27)]. Other examples include dipeptides such as 3-CF_3-C_6H_4-CO-Ala-Ala-NH-C_6H_4-NO_2 [$K_I = 4 \times 10^{-6}$M (28)], fatty acids such as oleic acid [$K_I = 9 \times 10^{-6}$M (29)], and sulfated polysaccharides such as Arteparon and pentosan polysulfate SP-54 [$K_I = 10^{-7}$-10^{-8}, uncompetitive inhibition with multiple binding (30, 31)]. None of these compounds appears to offer significant advantages as do HL elastase inhibitors.

Animal Studies

Several different types of synthetic elastase inhibitors have been used in animal studies of emphysema. These have included peptide chloromethyl ketones, furyl saccharine, and trifluoroacetyldipeptides. However, peptide chloromethyl ketones have been the most widely studied.

The chloromethyl ketone Ac-Ala-Ala-Pro-AlaCH_2Cl has been extensively studied in hamsters. Experimental emphysema was induced by intratracheal instillation of porcine pancreatic elastase. The inhibitor was administered intraperitoneally in divided doses before and immediately after instillation of the elastase (32). The inhibitor was shown to have significant antielastase activity *in vivo* and markedly decreased the extent of elastase-induced emphysema. In addition, it was shown that the development of emphysema was dependent on the dose of Ac-Ala-Ala-Pro-AlaCH_2Cl with emphysema being eliminated with 4.1 and 8.0 mg doses and markedly diminished with 1.1 mg (33).

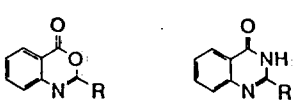

Fig. 7. Structure of a 2-substituted 4H-3,1-benzoxazin-4-one and a 2-substituted 4-quinazoline-one.

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The chloromethyl ketone Ac-Ala-Ala-Pro-AlaCH$_2$Cl was not effective when administered 60 min after exposure to elastase (34). A divided dose of 19 mg was injected intraperitoneally. In addition, this large dose was shown to produce a unique renal tubular nephropathy. In the previous studies when the inhibitor was administered concurrently with the elastase, no evidence of toxicity or kidney injury was observed with a similar dose. The mechanism of this *in vivo* toxicity is not yet known.

The chloromethyl ketone MeO-Suc-Ala-Ala-Pro-ValCH$_2$Cl has been shown to be orally active in protecting Swiss-Webster mice from elastase-induced emphysema (35). The mice were given a transoral dose of porcine pancreatic elastase and were given 300 to 400 μg of the chloromethyl ketone at times ranging from 2 h before instillation of the enzyme to 48 h after the enzyme. Mice that received the elastase inhibitor within 15 min before instillation of elastase were protected from development of emphysema and their lungs were completely free of alveolar deformation. However, no significant protection was afforded by treatment with MeO-Suc-Ala-Ala-Pro-ValCH$_2$Cl 1 h or more before the challenge with the enzyme or at any time after the challenge.

The chloromethyl ketone Suc-Ala-Ala-Pro-ValCH$_2$Cl has been shown by morphometric and physiologic measurements to moderate elastase-induced emphysema in hamsters (36). When 0.5 mg of the inhibitor in saline was injected intratracheally 1 hr before intratracheal injection of porcine pancreatic elastase, the hamsters did not develop emphysema. When the inhibitor was injected 1 hr after the elastase, the severity of emphysema was reduced by approximately 50% compared with control animals receiving saline 1 hr after elastase. The Suc-Ala-Ala-Pro-ValCH$_2$Cl was ineffective when administered intratracheally 4 h after the elastase.

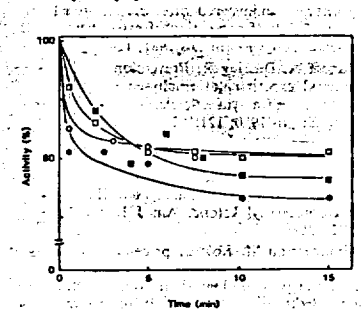

Fig. 8. The effect of elastin on the elastase catalyzed hydrolysis of a synthetic elastase substrate. Elastin (2 mg) was added to a solution of enzyme in 1 ml of saline (0.85% NaCl, 0.02% $CaCl_2$, 0.02% KCl, and 0.002% $NaHCO_3$) at room temperature. At appropriate intervals an aliquot (50 μl) of the suspension was assayed for elastase activity using MeO-Suc-Ala-Ala-Pro-Val-NA (2 mM) as a substrate in 2 ml of 0.1 M Hepes buffer at pH 7.5 containing 0.5 M NaCl and 10% DMSO. HL elastase (2.6×10^{-7} M) was studied with bovine ligamentum nuchae elastin (*open circles*) and dog lung elastin (*closed circles*). PP elastase (1.2×10^{-8} M) was studied with bovine ligamentum nuchae elastin (*open squares*) and dog lung elastin (*closed squares*).

TABLE 1

EFFECT OF ELASTIN ON THE RATE OF INHIBITION OF HL AND PORCINE PANCREATIC ELASTASE BY PEPTIDE CHLOROMETHYL KETONES

Inhibitor	Concentration (μM)	$k_{obs}/[I]$ ($M^{-1}s^{-1}$) No Elastin	Bovine Elastin	Dog Lung Elastin
	Porcine Pancreatic Elastase			
Ac-Ala-Ala-Pro-AlaCH$_2$Cl	4.1	366	181	44
Suc-Ala-Ala-Pro-ValCH$_2$Cl	1.5	685	274	132
MeO-Suc-Ala-Ala-Pro-ValCH$_2$Cl	1.9	630	249	87
	HL Elastase			
Ac-Ala-Ala-Pro-AlaCH$_2$Cl	81	7.4	4.3	7.2
Suc-Ala-Ala-Pro-ValCH$_2$Cl	0.31	1490	198	40
MeO-Suc-Ala-Ala-Pro-ValCH$_2$Cl	0.39	1550	125	71

Elastin (2 mg) was added to 1 ml of elastase in saline (HL elastase, 2.6×10^{-7} M; PP elastase, 1.2×10^{-8} M). After shaking for 10 min, a solution of the chloromethyl ketone in saline was added. At appropriate intervals, aliquots of the suspension were removed and assayed for residual elastase activity using MeO-Suc-Ala-Ala-Pro-Val-NA as a substrate. The k_{obs} was obtained from a plot of ln velocity versus time.

To explore the reasons for the ineffectiveness of peptide chloromethyl ketones when administered after the dose of elastase, we examined the reaction of elastase with substrate and inhibitors in the presence of elastin. When HL elastase or porcine pancreatic elastase is added to excess elastin, the ability of the enzyme to hydrolyze a small synthetic substrate is only slightly diminished (figure 8). This indicates that the elastase molecules bound to elastin are slightly less available to the substrate or that a slight alteration in the active site conformation has occurred. In addition, binding of the elastase to the elastin is complete within a few minutes.

Rates of inhibition of elastase by peptide chloromethyl ketones were then measured in the presence and absence of elastin (table I). In all cases, the chloromethyl ketone elastase inhibitors examined were much less effective at inhibiting elastase preabsorbed onto elastin. Indeed, with the chloromethyl ketones used in the animal emphysema models (porcine pancreatic elastase induced), inhibition rates were decreased by two-to eightfold. Even larger rate reductions were observed with HL elastase preabsorbed onto elastin. These low rates of inhibition may explain the ineffectiveness of peptide chloromethyl ketone elastase inhibitors in the animal models when administered after the dose of elastase. After the elastase has had an opportunity to bind to lung elastin, it is much harder to inhibit. It would be interesting to investigate whether all classes of elastase inhibitor exhibit this type of behavior. In future therapeutic protocols, it may be necessary to maintain a constant presence of elastase inhibitor in order to knock out the enzyme before it can interact with elastin.

Only a few other classes of elastase inhibitors have been studied in animal models. Using the papain-induced emphysema model in hamsters, chaulmoogric acid, furoyl saccharin, the azapeptide Ac-Ala-Ala-Pro-NHN(CH$_3$)CO-OCH(CH$_3$)CONH$_2$, and elastatinal have been shown to be effective inhibitors (37). The dipeptide CF$_3$CO-Lys-Ala-NHC$_6$H$_5$ and the chloromethyl ketone CF$_3$CO-AlaCH$_2$Cl have been tested in the porcine pancreatic elastase-induced emphysema model in hamsters (38). The chloromethyl ketone gave significant partial protection against the emphysematous lesions, but no protection was obtained with the dipeptide. This is an excellent *in vitro* elastase inhibitor and possibly it is cleaved at the Lys residue *in vivo* by some trypsin-like enzyme. Interestingly, it has been reported in an abstract that α_1-protease inhibitor or elastatinal were partially effective at suppressing emphysematous lesions in the hamster-elastase model when the inhibitors were administered 6 days after the elastase instillation (39). This observation should be confirmed independently by other investigators because other types of inhibitors (peptide chloromethyl ketones) have been shown to be ineffective at such a long interval after elastase instillation.

Prospects

It now appears likely that HL elastase is primarily responsible for the destruction of lung tissue observed in pulmonary emphysema. Other enzymes such as cathepsin G may act synergistically with HL elastase or contribute to the damage by other mechanisms. Thus synthetic elastase inhibitors have considerable potential for the treatment of emphysema. Indeed, several classes of compounds have already been shown to be active in animal models of emphysema.

At present, better elastase inhibitors are needed. They should have a high degree of selectivity and minimal side reactions or toxic effects. In addition, more studies are needed on the effect of elastase inhibitors on a variety of animal emphysema models.

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More toxicity data on some of the compounds already tested also are needed. However, the overall prospects are quite good for the development of a synthetic elastase inhibitor for use in humans in the near future.

It is clear that a synthetic elastase inhibitor would be a useful therapeutic agent. Further progress in the field simply awaits the development of a more ideal inhibitor structure.

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